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Nanotoxicology

State-of-the-Art and Future Research Needs

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Nanotoxicology – State-of-the-Art and Future Research Needs

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Preface

The Institute of Environmental Medicine (IMM), a department at Karolinska Institutet, is an interdisciplinary research organization in the field of environmental medicine. At IMM, internationally recognized research in the fields of toxicology, environmental medicine, and epidemiology is conducted. IMM also provides science-based environmental risk assessments to governmental agencies in support of standards and regulations. Researchers at IMM are active in numerous projects funded by the European Commission, thus providing a wide network of international partners. In particular, IMM has played a leading role in the field of nanotoxicology, with participation in numerous projects in the Seventh Framework Programme, eg., FP7-NANOREG, and in Horizon 2020, as well as a number of national projects such as the MISTRA Environmental Nanosafety project, a collaboration between 5 Swedish universities. IMM researchers also participate in the 10-year GRAPHENE Flagship Project, focusing on effects on human health and the environment.

The purpose of the current report is to provide an overview of the state-of-the-art of nanotoxicology, with particular emphasis on hazard assessment of nanomaterials for human health; effects on other species in the natural environment are not discussed. We discuss epidemiology of fine and ultrafine particles as a backdrop for the subsequent evaluation of engineered nanomaterials, and we reflect on the lessons learned from the first decade or more of nanosafety research, including the potential role of the so-called bio-corona on the surface of nanomaterials. Specifically, we address the four main areas of material characterization, exposure assessment, hazard assessment, and risk assessment of nanomaterials. We also discuss emerging systems toxicology approaches with which to understand the biological effects of nanomaterials. Finally, we identify knowledge gaps and future research needs in nanosafety. The report is a collaboration between scientists from several different units at IMM, and the work has been coordinated by prof. Bengt Fadeel, chair of the expert panel of the national nanosafety platform and head of the unit of molecular toxicology.

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Abbreviations

ALI, air-liquid interface

AOP, adverse outcome pathway

BAL, bronchoalveolar lavage

BMD, benchmark dose

CNT, carbon nanotube

ECHA, European Chemicals Agency

ENM, engineered nanomaterial

HTS, high-throughput screening

IARC, International Agency for Research on Cancer

ISO, International Organization for Standardization

KE, key event

KEMI, Swedish Chemicals Agency

KET, key enabling technology

MIE, molecular initiating event

MOA, mode-of-action

NIOSH, National Institute for Occupational Safety and Health

NOAAs, nano-objects and their agglomerates and aggregates

OECD, Organization for Economic Co-operation and Development

OEL, occupational exposure limit

PBPK, physiologically based pharmacokinetic

PM, particulate matter

QSAR, quantitative structure-activity relationship

REACH, Registration, Evaluation, Authorization and Restriction of Chemicals

STIS, short-term inhalation study

WHO, World Health Organization

WPMN, Working Party on Manufactured Nanomaterials

Executive Summary

Nanotechnology harnesses the unique properties of materials at the nanoscale. It is generally believed that nano-enabled technologies will have a pervasive impact on society, and engineered nanomaterials or ENMs are commonly hailed as one of the elements of a new industrial revolution. In light of the increasing production and use of ENMs across the globe, it is an essential priority to address the safety of this expanding class of materials for human health and the environment. Thus, while significant investments in nanosafety research have been made in recent years, the knowledge regarding interactions of ENMs with living systems needs to be translated into a risk management framework to support safe and sustainable development of existing and emerging nanotechnologies.

The main challenges

The recent report on safe handling of nanomaterials (SOU 2013:70) commissioned by the Swedish Ministry of Environment and Energy emphasized that measures are needed to exploit the opportunities that ENMs provide while minimizing potential risks to human health and the environment. It was also highlighted that safety research and innovation must be integrated. We fully support this view. Indeed, we believe that a national plan for nanotechnology research and innovation in which safety assessment is integrated at every step of the innovation process is an urgent goal. It is also important to consider the global nanosafety landscape in this regard. The EU nanosafety cluster published a strategic research agenda in 2013 and emphasized, amongst other things, the importance of ensuring that all the relevant stakeholders including both European and global organizations (eg., OECD) are involved in setting research priorities, to ensure that unnecessary duplication of efforts is avoided. The so-called communities of research, initiated by the European Commission and the US Government's National Nanotechnology Initiative (NNI), represent one such example of international dialogue between scientists in the field of nanotechnology-related environmental, health, and safety research. However, the dialogue should encompass all stakeholders. Nanosafety cannot exist in a vacuum as there is no safety *per se*, only safety (or risk) in the context of innovation, production, and use.

The ProSafe White Paper published in 2017 as a joint effort of the FP7-NANOREG project and the Horizon2020 ProSafe project, highlighted that it remains difficult to come to conclusions regarding the risks of most nanomaterials and nano-enabled products, the main reason being that nanosafety research during the past decade has been predominantly "science-oriented" rather than "regulation-oriented". We agree, to some extent, with the conclusions in the latter report. Hence, it is correct that considerable investments have been made in nanosafety research, not least in the EU, which has led to a better understanding of the biological interactions of ENMs. Moreover, we also note that the nanotoxicology community has been quick to adopt new and emerging approaches including high-throughput screening and omics-based systems toxicology tools. We agree that the exponential increase in the number of papers on nanotoxicology during the past decade does not automatically translate into useful tools for risk assessment and regulation of nanomaterials. This may be due, in part, to a *communication* gap – and further efforts are needed to bridge this communication gap between researchers and other stakeholders including regulatory agencies and industry in order to remedy the situation. The national platform, SweNanoSafe, represents an important and illustrative example in this regard. However, the current situation may also be due to an *expectation* gap – researchers hope to gain a better understanding of the principles that govern the biological interactions of nano-scale materials, while regulators require first and foremost a firm basis on which to determine risk to human health and the environment. These two goals are not mutually exclusive, but a dialogue is needed between the different stakeholders including relevant funding agencies in order to calibrate the expectations of all the concerned parties. Thus, researchers must understand that the experimental study design needs to take into account the key regulatory questions, while regulators need to appreciate that fundamental research on nano-bio interactions takes time and that the onslaught of new nanomaterials is a real challenge, both from the point of view of risk assessment and from a basic science point of view. Thus, we would argue that what we need is a two-pronged approach, i.e., continued investments both in fundamental and applied (regulatory) nanosafety research – and a better dialogue between the two.

The present report

The present report on the state-of-the-art of nanotoxicology provides an overview of each of the fundamental aspects of nanosafety including material characterization, exposure assessment, hazard assessment, and risk assessment. The report is a collaboration between several scientists at the Institute of Environmental Medicine (IMM), a department at

Karolinska Institutet, and a leading institute in environmental health risk assessment. We provide a detailed assessment of the potential hazards posed by ENMs including effects on key organ systems such as the pulmonary system, cardiovascular system, skin, gastro-intestinal system, immune system, and central nervous system, and we discuss developmental/reproductive effects as well as the carcinogenicity of ENMs. We also discuss the role of the so-called bio-corona on the surface of ENMs, and we provide an overview of epidemiological studies of fine and ultrafine particles, as nanotoxicology as a discipline necessarily builds on traditional particle and fibre toxicology. Finally, we discuss the implementation of advanced *in vitro* and *in silico* approaches to evaluate ENMs.

The report concludes with a set of future research challenges organized under the four main headings: material characterization, exposure assessment, hazard assessment, and risk assessment. From this survey of the literature, and on the basis of ongoing research activities at the Institute of Environmental Medicine, we conclude that considerable progress is being made in nanotoxicology; however, further, concerted efforts are required at the national level to promote the safe handling of ENMs.

1. Introduction

1.1. Production and Use of Engineered Nanomaterials

Nanotechnology is viewed as one of the key enabling technologies (KETs) that will allow European industries to retain competitiveness and capitalize on new markets. In October 2011, the European Commission recommended the following definition of a nanomaterial: 'a *natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.'* (2011/696/EU). Several types of nanomaterials, such as titanium dioxide, carbon black, aluminium oxide, calcium carbonate, and amorphous silica are being produced for specific applications in significant quantities. Indeed, the production of nanoparticles of titanium dioxide and silicon dioxide has grown globally to millions of tonnes *per annum*. The industrial application of engineered nanomaterials (ENMs) spans numerous societal sectors including various consumer products, as illustrated in Figure 1. Non-intentional, anthropomorphic nanoparticles are generated from vehicle exhaust and environmental waste while naturally occurring nanoparticles may arise from corrosion or volcanic ejections. The present report focuses mainly on ENMs.

The Woodrow Wilson International Center for Scholars and the Project on Emerging Nanotechnologies created the Nanotechnology Consumer Products Inventory (CPI) in 2005 (http://nanotechproject.org). Vance et al. (2015) reported a several-fold increase in the number of consumer products containing ENMs 10 years later. Silver is the most frequently used nanomaterial in consumer products (435 products in the CPI, or 24%); however, 49% of the products (889) included did not provide the composition of the nanomaterial used in them. The authors pointed out that the development of standardized methods and metrics for nanomaterial characterization and labelling in consumer products can lead to greater understanding between key stakeholders in nanotechnology, including consumers, researchers, regulators, and industry (Vance et al., 2015).

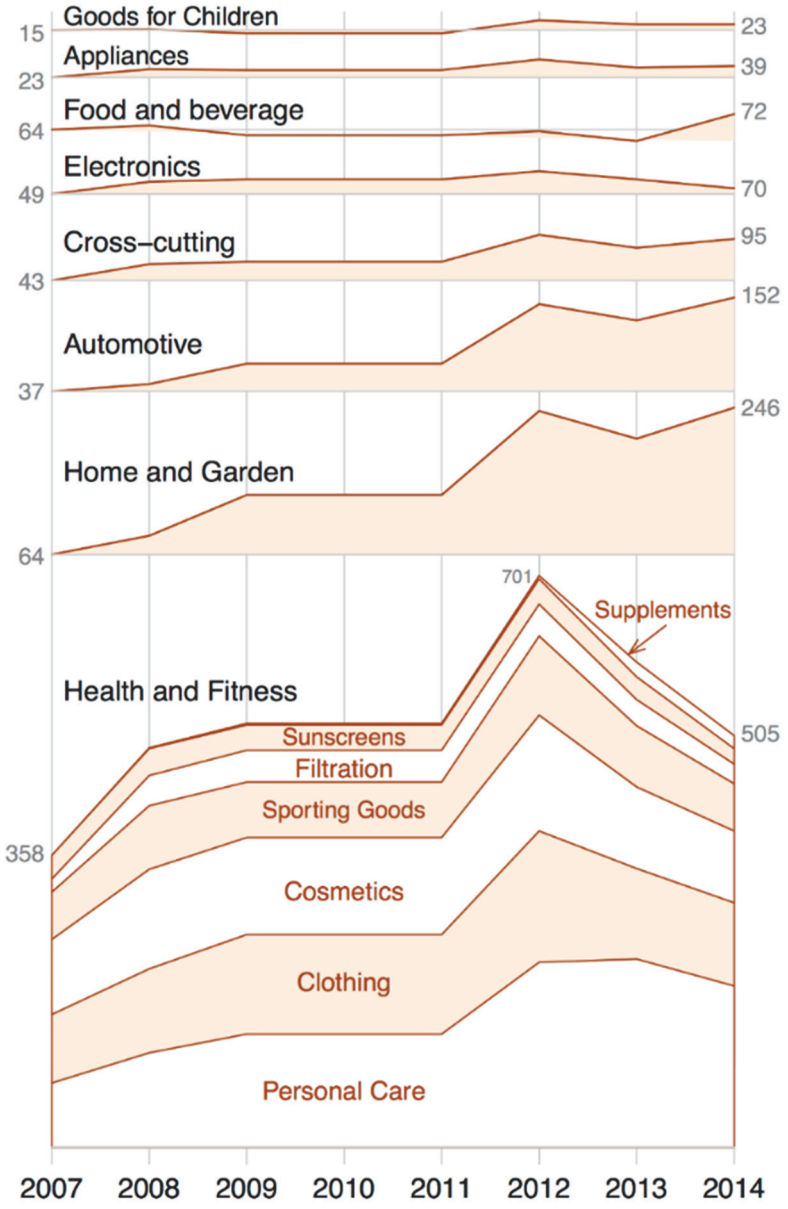


Figure 1. Nanomaterials in consumer products. Number of available products over time in each major category and in the health & fitness subcategories. From: Vance et al. 2015.

Hence, while nanotechnology is important for the development of new products, a major challenge is to evaluate the safety of the ENMs used in consumer products and other products and reduce human exposure during their life cycle from production to disposal. Nanoparticles can be designed in several forms depending upon their application, and alterations in the physicochemical properties of ENMs can alter the biological behavior. In fact, this is precisely why ENMs are being exploited, for instance, for biomedical applications. Hence, it is important to characterize ENMs in their pristine form and following release or biotransformation in order to understand the impact on humans and the

environment (Fadeel et al., 2015). The altered properties can change the way in which the material impact on humans and the environment and create unanticipated risks to health or the environment not seen for other chemicals.

The Swedish Ministry of Environment and Energy commissioned a report on safe handling of nanomaterials five year ago (SOU 2013:70) in which it was emphasized that measures are needed to exploit the opportunities that ENMs provide while minimizing the risks to human health and the environment. The large, EU-funded NANOREG and ProSafe projects, driven by the need to reduce uncertainty in the regulatory assessment of ENMs, recently put forward recommendations that are seen as necessary, feasible, effective, and cost-efficient (so-called 'no regret measures') (ProSafe, 2017), and called for innovation in risk assessment of ENMs. Thus, after a decade or more of nanotoxicology research, efforts are now being made to translate this knowledge into a science-based risk management framework to support safe and sustainable nanotechnology innovations.

References

Fadeel B, Fornara A, Toprak MS, Bhattacharya K. Keeping it real: the importance of material characterization in nanotoxicology. Biochem Biophys Res Commun. 2015;468(3):498-503.

Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF Jr, Rejeski D, Hull MS. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. Beilstein J Nanotechnol. 2015;6:1769-80.

ProSafe. The ProSafe White Paper. Towards a more effective and efficient governance and regulation of nanomaterials. 2017.

SOU 2013:70. Säker utveckling! – nationell handlingsplan för säker användning och hantering av nanomaterial. Betänkande av utredningen om nationell handlingsplan för säker användning och hantering av nanomaterial. Statens Offentliga Utredningar. (2013).

1.2. Hazard, Exposure, Risk: The Key Challenges

Nanotoxicology has emerged as a sub-discipline at the interface of toxicology and material science. The term ‘nanotoxicology’ was introduced more than a decade ago (Donaldson et al., 2004) to reflect the potential uniqueness of the physicochemical properties of nanoscale materials suggesting that their interactions with cells and tissues may give rise to novel and unpredictable effects, not anticipated on the basis of previous studies of larger particles. There has been an exponential increase in the number of studies concerned with the toxicity of nanomaterials, with a propensity of studies on mammalian systems, but an increasing number of studies also on the environmental impact (Krug, 2014) (Figure 2). Importantly, there is now a greater awareness of the importance of the physical form and chemical composition of these materials for the interaction with biological systems. Indeed, research conducted during the past decade has highlighted the importance of detailed material characterization in nanotoxicology (Maynard et al., 2011; Fadeel et al., 2013).

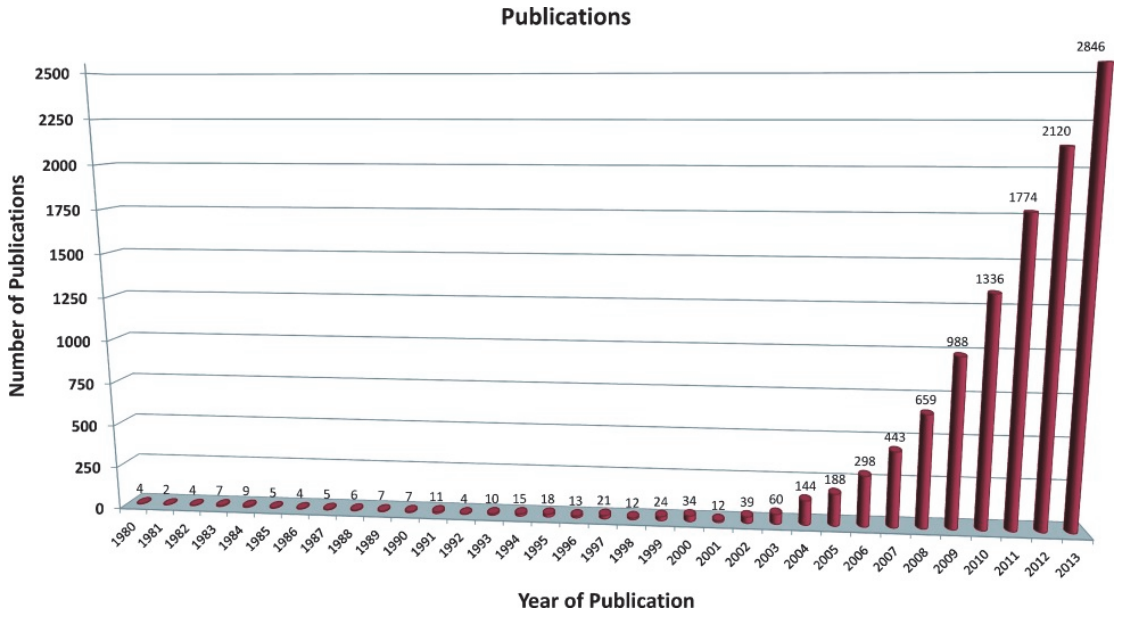


Figure 2. The number of scientific papers in nanotoxicology. From: Krug, 2014.

However, many challenges remain with regard to the potential risks of ENMs for human health and the environment. There is still a lack of information on the basic rules of nanomaterial interactions with biological systems; there is also a paucity of information on actual exposure levels and risk assessment currently relies on a case-by-case evaluation of nanomaterials. For some classes of nanomaterials, such as carbon nanotubes, a significant body of literature has emerged and some carbon nanotubes have been classified as being potential human carcinogens on

the basis of the available literature (Grosse et al., 2014). For other nanomaterials, including silver nanoparticles, the mechanism of toxicity can be plausibly explained by the release of toxic ions, a well-known mechanism of toxicity, and it has been argued that the scientific knowledge is sufficient to implement regulations for this class of materials (Nowack et al., 2011; Hansen & Baun, 2012). However, the question remains whether there are any novel or nano-specific effects for other classes of nanomaterials that warrant specific attention. The very fact that material physicochemical properties change at the nanoscale suggests that novel or unanticipated risks may also arise; indeed, as pointed out by Stark (2009) some nanoparticles resemble both molecules and solids: the combination of mobility (a property of classical molecules) with specific properties of a solid material (for example, magnetism; response to light, catalytic/chemical activity) allows delivery of the physical properties of solids into an organism. However, it is also important to acknowledge that some effects also conform to known effects of other fine and ultrafine particles or fibers, and it is not always necessary to invoke 'novel' effects.

In a seminal paper published more than one decade ago, Maynard et al. (2006) pointed out that "*the spectre of possible harm — whether real or imagined — is threatening to slow the development of nanotechnology unless sound, independent and authoritative information is developed on what the risks are, and how to avoid them*". The authors proposed five 'grand challenges' to stimulate research in the area of nanosafety (Maynard et al., 2006). However, due to the broad and all-encompassing nature of these challenges (eg., develop instruments to assess exposure, develop and validate methods to evaluate toxicity) it is difficult to properly evaluate whether progress has been made. Some authors have presented a rather dismal view, arguing that the vast majority of nanotoxicological studies are irrelevant from a risk assessment perspective as the studies were conducted with poorly characterized nanomaterials and/or at unrealistically high doses (Krug, 2014). Other authors have argued that mechanistic understanding of nanomaterial effects remains limited and suggested that there is, at present, no predictable pattern of toxicity for nanomaterials (Valsami-Jones et al., 2015). This may, at least in part, be due to the fact that nanomaterials cannot be grouped into one single class of materials displaying a uniform mechanism of action: some nanomaterials are fiber-like while other

"nanomaterials cannot be grouped into one single class of materials displaying a uniform mechanism of action"

nanomaterials dissolve into toxic ions, and some nanomaterials are evidently inert. Hussain et al. (2015) suggested that even though great strides have been made to advance the field, nanotoxicology is currently at a crossroads and faces a number of obstacles not associated with traditional toxicology (of particles and fibers). Some of the most pressing challenges include establishing characterization requirements, standardization of dosimetry, evaluating kinetic rates of ionic dissolution, improving *in vitro* to *in vivo* predictivity, and establishing safety exposure limits (Hussain et al., 2015). In addition, one may add that more information is needed on the exposure levels of ENM along the life cycle of a nanomaterial-enabled product, and occupational exposure limit values are needed. Notwithstanding, it is clear that key lessons have been learned not least with regard to the crucial importance of material characterization. Furthermore, new approaches in nanotoxicology including mechanism-based high-throughput screening (Nel et al., 2013) and systems toxicology methods to reveal novel biomarkers and pathways involved in the mode-of-action of nanomaterials (Riebeling et al., 2017) are gaining traction. The challenge is to translate this knowledge into useful tools for risk assessment of current and newly emerging nanomaterials.

References

Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ. Nanotoxicology. Occup Environ Med. 2004;61(9):727-8.

Fadeel B, Alenius H, Savolainen K. Nanotoxicology. Toxicology. 2013;313(1):1-2.

Grosse Y, Loomis D, Guyton KZ, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Scoccianti C, Mattock H, Straif K. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. Lancet Oncol. 2014;15(13):1427-8.

Hansen SF, Baun A. When enough is enough. Nat Nanotechnol. 2012;7(7):409-11.

Hussain SM, Warheit DB, Ng SP, Comfort KK, Grabinski CM, Braydich-Stolle LK. At the crossroads of nanotoxicology in vitro: past achievements and current challenges. Toxicol Sci. 2015;147(1):5-16.

Krug HF. Nanosafety research--are we on the right track? Angew Chem Int Ed Engl. 2014;53(46):12304-19.

Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdörster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle SS, Tran L, Walker NJ, Warheit DB. Safe handling of nanotechnology. Nature. 2006;444(7117):267-9.

Maynard AD, Warheit DB, Philbert MA. The new toxicology of sophisticated materials: nanotoxicology and beyond. Toxicol Sci. 2011;120 Suppl 1:S109-29.

Nel A, Xia T, Meng H, Wang X, Lin S, Ji Z, Zhang H. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc Chem Res. 2013;46(3):607-21.

Nowack B, Krug HF, Height M. 120 years of nanosilver history: implications for policy makers. Environ Sci Technol. 2011;45(4):1177-83.

Riebeling C, Jungnickel H, Luch A, Haase A. Systems biology to support nanomaterial grouping. Adv Exp Med Biol. 2017;947:143-171.
Stark WJ. Nanoparticles in biological systems. Angew Chem Int Ed Engl. 2011;50(6):1242-58.
Valsami-Jones E, Lynch I. Nanosafety. How safe are nanomaterials? Science. 2015;350(6259):388-9.

1.3. Epidemiology of Fine and Ultrafine Particles

There are currently no studies of long-term health effects in populations exposed to ENM. Existing cohorts of workers exposed to nanomaterials are currently too small to investigate chronic health effects (Canu et al 2016, Fatkhutdinova et al 2016). Occupational exposure to ENP concerns relatively small groups, and follow-up time is currently too short to allow conclusions regarding health effects like cancer or cardiovascular disease. However, there is a huge epidemiological literature on long-term effects of exposure to fine or ultrafine particles of other origin in the occupational and general (urban) environment. This literature may be of relevance to anticipate potential long-term health hazards from ENPs. In fact, as pointed out recently by a workshop of experts, there is now an opportunity to apply knowledge from nanotoxicology and use it to better inform particulate matter health risk research and *vice versa* (Stone et al., 2017).

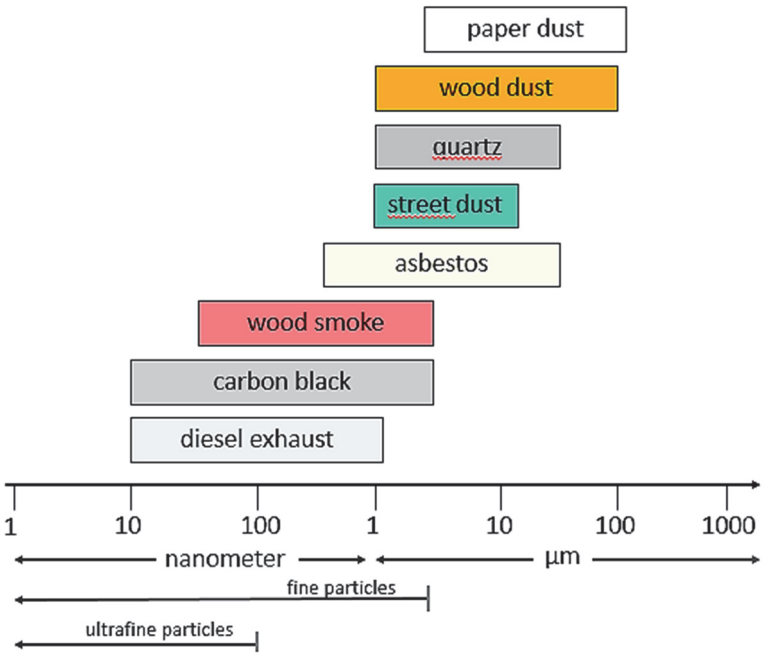


Figure 3. Sizes and sources of some fibres and particles.

Particle size ranges and sources for some particles and fibres occurring in the work environment or in urban air are presented in Figure 3. Generally, particles with an aerodynamic diameter below 1 µm are formed during combustion. A widespread exposure is diesel motor exhaust, particles being about 50 nm in diameter when generated, and later aggregating to form particles up to about 1 µm (Bockhorn 1994). Carbon black is an industrially produced combustion-generated particle used in laser printers,

rubber tyres, and in printing ink. Mechanically generated particles like street dust, crystalline silica (common in construction and stone industry), wood dust, or paper dust are generally larger than 1 μm in aerodynamic diameter. Asbestos is a naturally occurring fibre of various dimensions. Air-borne asbestos fibres in occupational settings may have a diameters down to well below 1 μm, and lengths up to or over 40 μm. Some of these particles are of special relevance in relation to potential health effect from exposure to engineered nanoparticles and fibres. For instance, diesel motor exhaust and carbon black are of special interest regarding health effects from spherical ENMs (of carbonaceous origin), while asbestos fibres are of special interest due to their similarity to carbon nanotubes (CNTs), in particular multi-walled CNTs. There is an extensive literature on urban air pollution and cardiovascular disease as well as cancer although the findings are not easily interpreted in relation to particle size (Stone et al., 2017). Epidemiological findings regarding cancer and cardiovascular disease in association with exposure to diesel exhaust, carbon black, asbestos, and urban air pollution are briefly reviewed below.

“there is an extensive literature on urban air pollution and cardiovascular disease as well as cancer”

Occupational exposure to diesel exhaust

There are numerous studies of occupational exposure to diesel exhaust and lung cancer, and a smaller number of studies on cardiovascular disease. Diesel exhaust is a complex mixture of particles and gases. In a diesel motor, the fuel is oxidized during combustion, forming larger molecules, in turn aggregating to particles, originally in a size range of about 50 nm. After emission, the particles are further aggregated and may form complexes up to about 1 μm in diameter (Bockhorn 1994). Diesel exhaust particles essentially consist of a core of carbon, to which inorganic and organic substances (including polycyclic aromatic hydrocarbons) are adsorbed (IARC, 2014). The currently most commonly used exposure indicator for diesel exhaust is elemental carbon, which represents the carbon core of the diesel particles with the adsorbed substances burnt off.

The epidemiological literature on diesel exhaust very consistently shows an increased risk of lung cancer in exposed groups. Based on sufficient evidence for carcinogenicity in humans as well as in experimental animals, diesel exhaust was classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) in 2012 (IARC, 2014). Which components of diesel exhaust that account for the

carcinogenic effect is less clear. In animal experiments, filtered diesel exhaust was not carcinogenic, indicating that the particle phase is important for the carcinogenicity (IARC, 2014). The cumulative exposure to elemental carbon was shown to correlate with lung cancer risk in several large epidemiological studies (Vermeulen et al., 2014). The technical development in diesel fuel and motors has reduced the particle content in the exhaust considerably, although it is not known if the cancer hazard has been reduced to the same extent. The Nordic Expert Group for hygienic threshold values recently proposed that an occupational exposure limit for diesel exhaust should be based on both elemental carbon and nitrogen dioxide, the latter substance being traditionally the most common indicator of diesel exhaust, albeit not carcinogenic in itself (NEG, 2016). Diesel exhaust is richer in content of both particles and nitrogen dioxide than gasoline exhaust. The literature on health effects from gasoline exhaust is scarce, as there are few exposure circumstances where gasoline exhaust exposure occurs without simultaneous exposure to diesel exhaust. Based on inadequate evidence in animals as well as in humans, gasoline exhaust was classified as possibly carcinogenic to humans (Group 2B) by the IARC (2014).

Cardiovascular disease in relation to occupational exposure to diesel exhaust has not been studied to the same extent as cancer. Mortality from cardiovascular diseases is reported in many epidemiological studies, although dose-response analyses are typically focused on lung cancer, leaving uncertainty about the conclusions regarding cardiovascular disease. Studies of US trucking industry workers, Swedish as well as US construction workers, a Finnish record-linkage study and a case-control study from Stockholm all support that diesel exhaust may increase the risk of cardiovascular disease. The Swedish Agency for Health Technology Assessment and Assessment of Social Services (SBU) recently evaluated the literature on cardiovascular disease and occupational exposure. The SBU concluded that there was moderately strong evidence for a causal association between occupational exposure to motor exhaust and the risk of heart disease, whereas the evidence for an association with stroke and hypertension was inadequate (SBU, 2017). The evidence for an increased risk of cardiovascular disease from occupational exposure to diesel motor exhaust is thus weaker than the evidence for an increased risk of lung cancer.

To summarize, there is strong evidence that nano-sized spherical particles generated by combustion are carcinogenic. Diesel exhaust is a complex mixture of particles and adsorbed substances, including the well-established carcinogens from the group of PAHs, and it is not known to which extent the effects on lung cancer and cardiovascular disease is

associated with the particles as such or if adsorbed organic or inorganic substances are required for a hazardous effect. Thus, a remaining question is if nano-sized particles represent a health hazard in themselves or if adsorbed substances are important for the health effect? Unfortunately, there are few such exposure circumstances, though epidemiological data on carbon black would have been helpful in this regard.

Carbon black

Carbon black is a nano-sized carbon particle that is commercially produced in large amounts. Carbon black has a much lower content of PAHs than diesel exhaust. Carbon black and carbon black extracts are carcinogenic to animals (IARC, 2010). Unfortunately, few epidemiological studies of carbon black exposure are available. Most influential in an evaluation of carcinogenicity made by the IARC were three cohort studies of carbon black production workers. One study concerned workers from 18 US carbon production facilities, one concerned five UK production facilities, and one was a cohort study from a production factory in Germany. While there was some, albeit inconsistent evidence for a carcinogenic effect in the German and UK studies, the US study gave no support for this. Carbon black was classified as possibly carcinogenic to humans (Group 2B) based on sufficient evidence for carcinogenicity in animals and inadequate evidence in humans (IARC, 2010). The potential effect on the cardiovascular system from exposure to carbon black was investigated in a recent meta-analysis of cardiac mortality in the three cohorts that formed the basis for the IARC evaluation. A small and statistically insignificant overall excess of death from acute myocardial infarction was found. Further investigation of the dose-response showed a non-significant association with cumulative dose (Morfeld et al., 2016). Taken together, evidence for an excess of lung cancer and cardiovascular disease in association with occupational exposure to carbon black is weak.

Asbestos fibres

The carcinogenicity of CNTs was evaluated by the IARC in 2014. The working group concluded that there were no epidemiological data available. Based on sufficient evidence for carcinogenicity in animals, MWCNT-7 was classified as possibly carcinogenic to humans (Group 2B), whereas the evidence for carcinogenicity of other single- and multi-walled CNTs was insufficient. (IARC, 2017). No epidemiological data have been published on CNTs after the IARC review. MWCNTs are biostable and have structural similarities to the well-known human carcinogen asbestos,

which has led to speculations regarding carcinogenic effects of MWCNTs. The literature on asbestos is rich, especially concerning lung cancer, although there are also studies investigating cardiovascular effects. Asbestos is a group of naturally occurring mineral silicate fibres. It is mined and used for instance for heat and electrical insulation, in asbestos cement, and a large number of other applications. Asbestos is carcinogenic to humans and produces tumors in the lung, pleura (mesothelioma), larynx, and ovary (IARC, 2012). There is also evidence for an increased incidence of colorectal and stomach cancer in asbestos exposed populations, although the evidence is not fully sufficient for these cancer sites (FIOH, 2014). The first epidemiological studies showing a carcinogenic effect of asbestos were published in the 1950s, but it took a very long time until exposures were reduced. The use of asbestos was banned in the EU in 2005, although asbestos is still mined and used in other parts of the world (Gustavsson, 2014). Asbestos is very biostable and is degraded slowly in the body (IARC, 2012). In contrast, man-made vitreous fibres (MMVF) (“insulation wool”, i.e., stone wool and glass wool) have been freed from suspicions of carcinogenicity in several large epidemiological studies. MMVF fibres are more soluble than asbestos, and this is considered a likely explanation for the lack of carcinogenicity of MMVF, despite similarities to asbestos in fibre dimensions (IARC, 2002). However, the simultaneously evaluated more biostable refractory ceramic fibres (RCF) were carcinogenic to animals. Long and thin fibres, i.e., fibres >=5-10 microns in length and <= 0.25 microns in width, have been postulated to be especially carcinogenic, the so-called Stanton or Lippmann hypothesis (Stanton, 1981; Lippmann, 1990). This hypothesis was mainly based on pleural instillation of asbestos fibres in animal pleura and development of mesothelioma. The relevance for cancer in humans have been questioned, and the IARC concluded that there was relatively weak epidemiological evidence to support this hypothesis (IARC, 2012). A later published epidemiological study specifically addressed the influence of asbestos fibre dimensions on lung cancer risk. Fibre lengths from <= 1.5 µ to >40 µ and widths from <= 0.25 µ to >3 µ were investigated. Lung cancer risk increased with fibre length and decreased with fibre width, supporting that long and thin fibres are more carcinogenic than short and thick fibres (Loomis 2010). The ‘fibre paradigm’ is considered by several researchers to be applicable to CNTs, based on animal data (Kuempel et al., 2017).

There is a large number of studies of asbestos-exposed workers in which the risk of cardiovascular disease has been reported. A recent review and metaanalysis included 16 cohort studies of asbestos exposed workers. The meta-SMR for death from cardiovascular disease was slightly but

statistically significantly elevated (SMR= 1.11, 95% CI 1.01-1.22), although the heterogeneity in risk between studies was high (Rong et al 2015). The Swedish Agency for Health Technology Assessment and Assessment of Social Services concluded that there was moderately strong evidence for an association between occupational exposure to asbestos and an increased risk of death from heart disease (SBU, 2017).

Urban air pollution

The main source of air pollution in large cities in the western world today is generated by traffic. In an historical perspective, and in other parts of the world, emission form heating and industrial processes may also be large contributors. Vehicle traffic generates both fine-particulate motor exhaust and coarser particles from wear of street lining and tyres, so-called street dust. The motor exhaust particles are generally below 1 µm in diameter while the street dust particles are larger than 1 µm, see Figure 3.

The first observations of urban air pollution and risk of death came from the serious London smog episode in 1952. Several years later, large studies with more precise exposure estimations as well as detailed individual data on potential confounders have established that urban air pollution is associated with an increased risk of both cardiovascular disease (Newby et al., 2015) and cancer (IARC, 2016). The most commonly used exposure indicator of urban air pollution is particulate matter with an aerodynamic diameter less than 2.5 µm ($PM_{2.5}$). A meta-analysis of studies of long-term effects of urban air pollution estimated that the risk of death from cardiovascular diseases increased by 11% (95% CI 5, 16%) for every increase of 10 µg/m^3 of PM2.5 (Hoek et al., 2013). Thus, it is well established that urban air pollution is associated with long-term health effects such as cardiovascular disease and lung cancer. However, the interpretation in terms of health effects of particles of various sizes is not clear, since urban air pollution is a mixture of motor (mainly diesel) exhaust and street dust, with potential contribution also from industrial emissions and heating, depending on city characteristics. The exposure to street dust generated by traffic is so closely correlated to motor exhaust exposure that it is hard to disentangle the effects of these particle types from epidemiological data on long-term health effects of urban air pollution.

Concluding remarks

The epidemiology on occupational exposure to particles from diesel motor exhaust, which are in the submicron range, shows that these are carcinogenic to humans. It is uncertain, though, to which extent this effect is due to the particles as such (elemental carbon) or due to adsorbed substances such as PAHs. The epidemiology on cardiovascular disease and diesel exhaust is less conclusive, but clearly indicative of the existence of such an effect. Epidemiological studies of urban air pollution show associations both with cancer and cardiovascular disease, although it is not known if this is due to ultrafine particles from motor exhaust or to the larger street dust particles. The literature on carcinogenic potency of asbestos, man-made vitreous fibres and refractory ceramic fibres indicate that the cancer hazard is greater from long, thin and highly biopersistent fibres. It remains to be understood whether MWCNTs are so similar to asbestos in structure and characteristics so as to pose a potential cancer hazard.

References

Bockhorn H. Soot formation in combustion. Mechanisms and models. Springer Verlag, Berlin 1994, p. 4.

Canu IG, et al, the French program for epidemiological surveillance of workers potentially exposed to engineered nanomaterials: the state of play after first 18 months of operation. Abstract presented at the EPICOH in Barcelona 2016. Occup Environ Med 2016 73: A61

Fatkhutdinova LM, Khaliullin TO, Vasil'yeva OL, Zalyalov RR, Mustafin IG, Kisin ER, Birch ME, Yanamala N8, Shvedova AA. Fibrosis biomarkers in workers exposed to MWCNTs. Toxicol Appl Pharmacol. 2016;299:125-31.

FIOH. Asbestos, asbestosis and cancer. Helsinki criteria for diagnosis and attribution. Finnish Institute of Occupational Health (FIOH), Helsinki, 2014.

Gustavsson P. Strategies for primary prevention of occupational cancer. In: Anttila S & Boffetta P (Eds). Occupational Cancers. Springer Verlag, London 2014, p. 565-572

Hoek G, Krishnan RM, Beelen R, Peters A, Ostro B, Brunekreef B, Kaufman JD. Long-term air pollution exposure and cardio-respiratory mortality: a review. Environ Health. 2013;12(1):43.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 81. Man-made Vitreous Fibers. The International Agency for Research on Cancer, Lyon, France, 2002

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 93. Carbon Black, Titanium Dioxide, and Talc. The International Agency for Research on Cancer, Lyon, France, 2010

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 100C Arsenic, Metals, Fibres and Dusts. The International Agency for Research on Cancer, Lyon, France, 2012

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 105 Diesel and Gasoline Engine Exhausts and Some Nitroarenes, The International Agency for Research on Cancer, Lyon, France, 2014.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 109. Outdoor air pollution. The International Agency for Research on Cancer, Lyon, France 2016.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 111. Some Nanomaterials and Some Fibres. The International Agency for Research on Cancer, Lyon, France, 2017.

Kuempel ED, Jaurand MC, Møller P, Morimoto Y, Kobayashi N, Pinkerton KE, Sargent LM, Vermeulen RC, Fubini B, Kane AB. Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans. Crit Rev Toxicol. 2017;47(1):1-58.

Lippmann M. Effects of fiber characteristics on lung deposition, retention, and disease. Environ Health Perspect. 1990;88:311-7.

Loomis D, Dement J, Richardson D, Wolf S. Asbestos fibre dimensions and lung cancer mortality among workers exposed to chrysotile. Occup Environ Med 2010;67:580-584.

Newby DE, Mannucci PM, Tell GS, Baccarelli AA, Brook RD, Donaldson K, Forastiere F, Franchini M, Franco OH, Graham I, Hoek G, Hoffmann B, Hoylaerts MF, Künzli N, Mills N, Pekkanen J, Peters A, Piepoli MF, Rajagopalan S, Storey RF; Expert position paper on air pollution and cardiovascular disease. Eur Heart J. 2015;36(2):83-93.

Morfeld P, Mundt KA, Dell LD, Sorahan T, McCunney RJ. Meta-analysis of cardiac mortality in three cohorts of carbon black production workers. Int J Environ Res Public Health. 2016;13(3). pii: E302.

Nordiska expertgruppen för gränsvärdesdokumentation (NEG). Vol 149. Diesel Engine Exhaust. Arbete och Hälsa (Work and Health) No 2016;49(6). Gothenburg University.

SBU. Arbetsmiljöns betydelse för hjärt-kärlsjukdom. Exponering för kemiska ämnen. Rapport nr 261 SBU – Statens beredning för medicinsk och social utvärdering, 2017.

Stanton, M.F., Layard, M., Tegeris, A., Miller, E., Margaret, M., Kent, E., 1981. Relation of particles dimension to carcinogenicity in amphibole asbestoses and fibrous minerals. JNCI 67, 965–975.

Stone V, Miller MR, Clift MJD, Elder A, Mills NL, Møller P, Schins RPF, Vogel U, Kreyling WG, Alstrup Jensen K, Kuhlbusch TAJ, Schwarze PE, Hoet P, Pietroiusti A, De Vizcaya-Ruiz A, Baeza-Squiban A, Teixeira JP, Tran CL, Cassee FR. Nanomaterials *versus* ambient ultrafine particles: an opportunity to exchange toxicology knowledge. Environ Health Perspect. 2017;125(10):106002.

Vermeulen R, Silverman DT, Garshick E, Vlaanderen J, Portengen L, Steenland K. Exposure-response estimates for diesel engine exhaust and lung cancer mortality based on data from three occupational cohorts. Environ Health Perspect. 2014;122(2):172-7.

1.4. Physico-Chemical Properties and their Link to Toxicity

The major physicochemical properties of ENMs namely, size distribution, surface charge, stability in dispersion, shape, morphology/ porosity/ crystallinity, and surface functionality dictate how the nanomaterials interact with biological systems. However, these physicochemical properties are intertwined and they may regulate each other. Key material properties, and the aging or transformation of ENM, are discussed below.

Size and shape

Size determines whether a material is a nanomaterial. With decreasing size there is an increase in reactivity which is mediated by a larger percentage of atoms found at the surface (Auffan et al., 2009). This reactivity can enhance for example optical and catalytic properties on one hand but could also drive an increase in toxicity on the other hand (Figure 4).

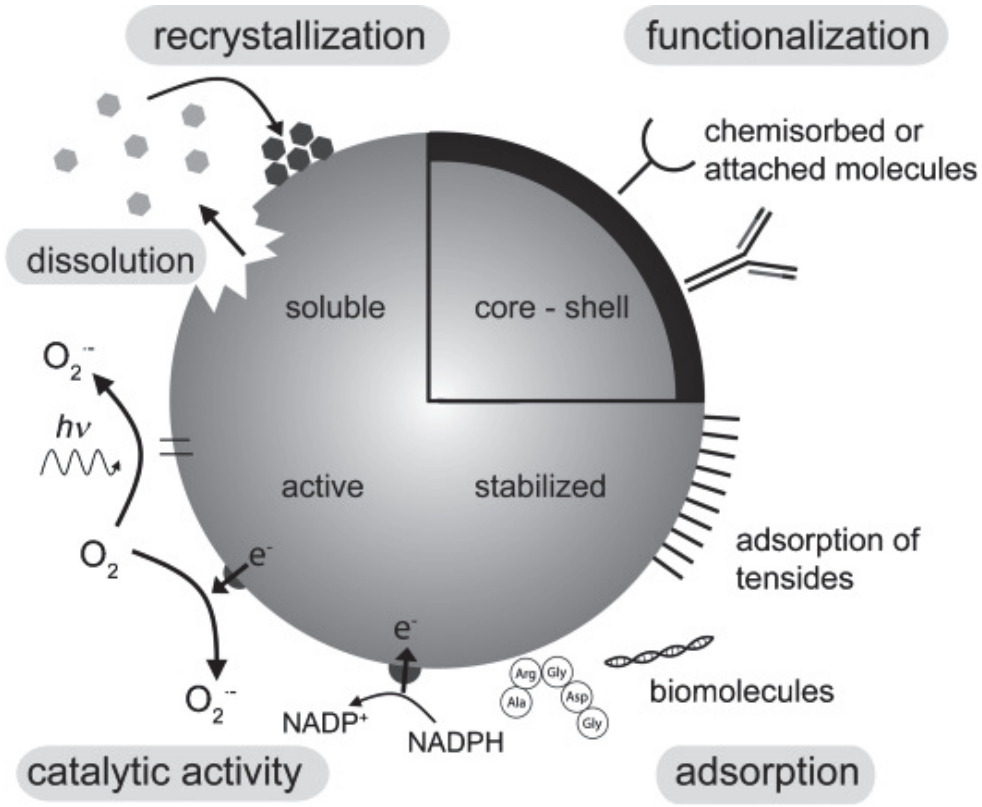


Figure 4. Surface modification or functionalization of nanoparticles alters the dissolution and degradation and plays a role for biological effects. Nanoparticles may adsorb macromolecules which may affect cell interactions. Catalytic activity alters the interaction of the particles with their surroundings. ENMs may dissolve with release of reactive ions and they could also recrystallize in a biological system. From: Stark, 2011.

Size is related to surface area which increases proportionally to the decrease in size (Hubbs et al., 2013). In addition, size can trigger novel interactions with biological systems in terms of cellular uptake and biodistribution, which can be different as compared to microparticles (Donaldson and Poland, 2013). It is well-established that smaller particles can deposit deeper in the lungs (Oberdörster et al., 2005) and have a higher translocation across the lung-blood barrier (Kreyling et al., 2009; Sadauskas et al., 2009). ENMs can also modulate cell signaling in innate immune cells in a size-dependent manner (Tsai et al., 2012). However, when suspended, nanoparticles display a different size distribution due to aggregation, agglomeration and dissolution. There is thus a distinction between the primary particle size and the size of nanomaterials in dispersion where they form agglomerates and aggregates. Primary particle size is usually determined by transmission electron microscopy (TEM) whereas the hydrodynamic size of (spherical) nanomaterials in dispersion is evaluated mainly by using dynamic light scattering (DLS) techniques.

Notably, *shape* is also a potentially important determinant of nanomaterial biological effects – in line with effects of other fibre-like materials (see preceding section). For instance, long and rigid MWCNTs were shown to elicit ‘asbestos-like’ effects *in vitro* as well as *in vivo* while short or tangled MWCNTs were inert (Poland et al., 2008; Palomäki et al., 2011). Furthermore, in a comparative study of Ag nanospheres and Ag nanowires, shape was shown to be an important factor in determining *in vitro* toxicity (Stoehr et al., 2011). Similarly, Hamilton et al. (2009) found that long TiO_2 nanobelts induced ‘asbestos-type’ toxicity in macrophages.

Surface charge

Surface charge acquired by ENMs in a colloidal suspension is quantified in terms of zeta potential. The zeta potential is determined by the electric potential created between the surface of the particle and the dispersion medium and is indicative of the surface charge of the nanoparticles (Cho *et al.*, 2012). The surface charge can influence the stability of the nanoparticles in dispersion as well as the interactions with cellular membranes which in turn can influence the toxic outcome. Moreover, a positive charge on the surface of ENMs may promote close interactions with negatively charged biomolecules such as DNA. However the surface charge is bound to change in biological environments when the particle is coated with biomolecules (so-called bio-corona formation). It was reported that cationic particles are more likely to disrupt the structure of the cell membrane as compared to anionic particles (Fröhlich, 2012). The

zeta potential in acidic conditions (similar to the lysosome) for low-soluble metal and metal oxide nanoparticles was a good predictor of lung inflammation in rodents (Cho et al., 2012). Moreover, positively charged nanoparticles (eg., amine terminated) can modulate the lysosomal proton pump through sequestration of protons, ultimately leading to the rupture of lysosomes and the release of ENMs into the cytosol, with spillage of lysosomal contents including cathepsins, and the induction of cell death.

Stability / degradation

Stability in dispersion is an important aspect that needs to be evaluated when working with nanoparticles *in vitro*. Particle agglomeration and aggregation can be triggered by changes in the zeta potential when nanoparticles are placed into a biological environment and depends both on the properties of the particles and on the properties of the dispersion medium. These phenomena are often followed by particle sedimentation that in turn will determine the delivered dose (i.e., the dose that reaches the desired target) which can differ dramatically from the nominal dose (i.e. the theoretical dose administered) (Kong et al., 2011). Determining the delivered dose is directly related to the cellular uptake (and often, not always, with toxic outcome) and is particularly important since most of the *in vitro* models use up-right culture setups. Indeed, it was reported that the uptake of gold nanoparticles was higher under upright *versus* inverted cell culture conditions for particles with high sedimentation velocity as compared to diffusion velocity (Cho et al., 2011). The sedimentation can be either determined experimentally (using, e.g., DLS approaches under kinetic conditions) or can be estimated using approaches such as the 'In vitro Sedimentation, Diffusion and Dosimetry' (ISDD) model. The ISDD model predicts the kinetics of nanoparticles in the dispersion medium and takes into account parameters such as hydrodynamic particle size, agglomeration state, particle density, temperature, medium height, medium viscosity and density (Hinderliter et al., 2010). Solubility can directly influence the size of the nanoparticles in dispersion and can play a role in the toxic outcome. For example, for highly soluble metal nanoparticles, inflammation is triggered by the release of toxic ions that destabilize the lysosomal membrane (Cho et al., 2012). Finally, the *degradation* of certain CNTs can be viewed as a special case of nanomaterial solubility. Oxidized, but not pristine, single-walled CNTs are susceptible to enzymatic degradation (Allen et al., 2009) while multi-walled CNTs are much more resistant to such degradation (Russier et al., 2011). For a recent review on biodegradation, refer to: Bhattacharya et al. (2016).

Porosity / crystallinity

Porosity may also dictate the interaction of nanomaterials with their surrounding biological environment. Mesoporous SiO_2 nanoparticles were observed to be less hemolytic and toxic than their non-porous counterparts possibly due to the absence of silanol groups on the surface of the nanoparticles (Lin and Haynes, 2010; Rabolli et al., 2010). However, this was found to be reversed through ageing due to collapse of the pore structures with passage of time (Lin and Haynes, 2010). Therefore, it is important to determine the shape and porosity of the nanomaterials using, for instance, electron microscopy, and Brunauer–Emmett–Teller (BET) techniques. *Crystallinity* of ENMs has a profound effect on their behavior in cellular environment. Using TiO_2 nanoparticles, Jiang et al. (2008) showed that crystalline structure of nanoparticles plays an important role in the generation of reactive oxygen species (ROS). The highest amount of ROS was generated by the amorphous form of TiO_2 nanoparticle, followed by anatase, and then anatase/rutile mixtures, and lowest for rutile samples (Jiang et al., 2008). Thus, measuring the intrinsic activity of the ENMs based upon their crystalline structure is important as they can induce high surface reactivity towards cellular systems and induce toxicity either through direct interactions or through generation of acellular/intracellular ROS. The most effective method for measurement of crystallinity is through X-ray diffraction (XRD) analysis. SiO_2 nanoparticles show reactivity based upon their surface functional group silanol (-OH), that depends upon the crystal structure of the nanoparticle itself (Shi et al., 2012; Zhang et al., 2012). For fumed silica, a positive correlation of toxicity with hydroxyl concentration and potential to generate ROS was observed whereas colloidal silica was nontoxic (Zhang et al., 2012).

Ageing / biotransformation

Ageing of nanomaterials can occur during long-duration storages under ambient conditions due to temperature fluctuations, reactions with the atmospheric oxygen and reactions with liquid the nanoparticles are suspended in. The ageing process can change the surface functional groups present on the surface of the nanoparticles, affect their shapes, porosity and thus change their overall activity within a biological environment. Using CuO nanoparticles, it was found that the ageing process had a profound effect on their basic physicochemical properties such as, increase in the average particle size, shift in the size distribution as a result of coalescence and coarsening under ambient conditions of temperature and relative humidity size distribution, and the absence of Cu(I) phase in the aged nanoparticles (Mudunkotuwa et al., 2012).

Moreover, environmental and biological fate may change as a result of the ageing process of the nanoparticles, but this is an under-explored field. Ag nanoparticles following 6 months storage were found to give increased biological responses and toxicity in A549 cells that was related to their initial native surface charge with the positively charged nanoparticle giving highest toxicity and neutral charged nanoparticle the least toxicity (Izak-Nau et al., 2015). It is therefore important to determine the functionality of nanoparticles and their toxicity as a function of ageing. It is also important to consider that nanoparticles may undergo biotransformation *in situ* upon introduction into a living organism or the environment. Liu et al. (2012) performed detailed studies of the biotransformation of silver nanoparticles in biological environments. According to these authors, argyrial silver deposits are not translocated Ag nanoparticles, but rather secondary particles formed by partial dissolution in the GI tract followed by ion uptake, systemic circulation as organo-Ag complexes, and immobilization as zerovalent Ag nanoparticles by photoreduction in light-exposed skin. Using an array of analytical techniques including high-resolution electron microscopy, CeO_2 nanoparticles were recently shown to be processed differently in the spleen than in the liver in exposed rats (Graham et al., 2018).

References

Allen BL, Kotchey GP, Chen Y, Yanamala NV, Klein-Seetharaman J, Kagan VE, Star A. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. J Am Chem Soc. 2009;131(47):17194-205.

Auffan, M., J. Rose, J.-Y. Bottero, G. V. Lowry, J.-P. Jolivet, M. R. Wiesner. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nat Nanotechnol. 2009;4(10): 634-641.

Bhattacharya K, Mukherjee SP, Gallud A, Burkert SC, Bistarelli S, Bellucci S, Bottini M, Star A, Fadeel B. Biological interactions of carbon-based nanomaterials: from coronation to degradation. Nanomedicine. 2016;12(2):333-51.

Cho, E. C., Q. Zhang and Y. Xia. The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles. Nat Nanotechnol. 2011;6(6): 385-391.

Cho, W.S., R. Duffin, F. Thielbeer, M. Bradley, I. L. Megson, W. MacNee, C. A. Poland, C. L. Tran, K. Donaldson. Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal-oxide nanoparticles. Toxicol Sci. 2012;126(2):469-477.

Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part Fibre Toxicol. 2010;7:5.

Donaldson, K. and C. A. Poland. Nanotoxicity: challenging the myth of nano-specific toxicity. Curr Opin Biotechnol. 2013;24(4): 724-734.

Elias Z., Poirot O., Daniere M.C., Terzetti F., Marande A.M., Dzwigaj S., Pezerat H., Fenoglio I., Fubini B. Cytotoxic and transforming effects of silica particles with

different surface properties in Syrian hamster embryo (SHE) cells, Toxicol In Vitro. 2000;14:409–422.

Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. International Journal of Nanomedicine. 2012;7:5577-5591.

Fubini B., Zanetti G., Altilia S., Tiozzo R., Lison D., Saffiotti U., Relationship between surface properties and cellular responses to crystalline silica: studies with heat-treated cristobalite, Chem Res Toxicol. 1999;12:737-45.

Graham UM, Yokel RA, Dozier AK, Drummy L, Mahalingam K, Tseng MT, Birch E, Fernback J. Analytical high-resolution electron microscopy reveals organ-specific nanoceria bioprocessing. Toxicol Pathol. 2018;46(1):47-61.

Hamilton RF, Wu N, Porter D, Buford M, Wolfarth M, Holian A. Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity. Part Fibre Toxicol. 2009;6:35.

Hinderliter, P. M., K. R. Minard, G. Orr, W. B. Chrisler, B. D. Thrall, J. G. Pounds and J. G. Teeguarden. ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. Part Fibre Toxicol. 2010;7(1): 36.

Hubbs, A. F., L. M. Sargent, D. W. Porter, T. M. Sager, B. T. Chen, D. G. Frazer, V. Castranova, K. Sriram, T. R. Nurkiewicz, S. H. Reynolds, L. A. Battelli, D. Schwegler-Berry, W. McKinney, K. L. Fluharty and R. R. Mercer. Nanotechnology: toxicologic pathology. Toxicol Pathol. 2013;41(2):395-409.

Izak-Nau E, Huk A., Reidy B., Uggerud H., Vadset M., Eiden S., Voetz M., Himly M., Duschl A., Dusinska M., Lynch I., Impact of storage conditions and storage time on silver nanoparticles' physicochemical properties and implications for their biological effects. RSC Adv., 2015;5:84172-84185.

Jiang J., Oberdörster G., Elder A., Gelein R., Mercer P., Biswas P., Does nanoparticle activity depend upon size and crystal phase? Nanotoxicology. 2008;2:33-42.

Kong, B., J. H. Seog, L. M. Graham and S. B. Lee. Experimental considerations on the cytotoxicity of nanoparticles. Nanomedicine (Lond). 2011;6(5):929-941.

Kreyling, W. G., M. Semmler-Behnke, J. Seitz, W. Scymczak, A. Wenk, P. Mayer, S. Takenaka, G. Oberdörster. Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal Toxicol. 2009; 21(Suppl 1):55-60.

Lin YS, Haynes CL. Impacts of mesoporous silica nanoparticle size, pore ordering, and pore integrity on hemolytic activity. J Am Chem Soc. 2010;132(13):4834-42.

Liu J, Wang Z, Liu FD, Kane AB, Hurt RH. Chemical transformations of nanosilver in biological environments. ACS Nano. 2012;6(11):9887-99.

Mudunkotuwa IA, Pettibone JM, Grassian VH. Environmental implications of nanoparticle aging in the processing and fate of copper-based nanomaterials. Environ Sci Technol. 2012;46(13):7001-10.

Palomäki J, Välimäki E, Sund J, Vippola M, Clausen PA, Jensen KA, Savolainen K, Matikainen S, Alenius H. Long, needle-like carbon nanotubes and asbestos

activate the NLRP3 inflammasome through a similar mechanism. ACS Nano. 2011;5(9):6861-70.

Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, Stone V, Brown S, Macnee W, Donaldson K. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol. 2008;3(7):423-8.

Rabolli V, Thomassen LC, Princen C, Napierska D, Gonzalez L, Kirsch-Volders M, Hoet PH, Huaux F, Kirschhock CE, Martens JA, Lison D. Influence of size, surface area and microporosity on the in vitro cytotoxic activity of amorphous silica nanoparticles in different cell types. Nanotoxicology. 2010;4(3):307-18.

Russier J, Ménard-Moyon C, Venturelli E, Gravel E, Marcolongo G, Meneghetti M, Doris E, Bianco A. Oxidative biodegradation of single- and multi-walled carbon nanotubes. Nanoscale. 2011;3(3):893-6.

Sadauskas, E., N. R. Jacobsen, G. Danscher, M. Stoltenberg, U. Vogel, A. Larsen, W. Kreyling, H. Wallin. Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation. Chem Central J. 2009;3(1):1-7.

Shi J, Karlsson HL, Johansson K, Gogvadze V, Xiao L, Li J, Burks T, Garcia-Bennett A, Uheida A, Muhammed M, Mathur S, Morgenstern R, Kagan VE, Fadeel B. Microsomal glutathione transferase 1 protects against toxicity induced by silica nanoparticles but not by zinc oxide nanoparticles. ACS Nano. 2012;6(3):1925-38.

Stark WJ. Nanoparticles in biological systems. Angew Chem Int Ed Engl. 2011;50(6):1242-58.

Stoehr LC, Gonzalez E, Stampfl A, Casals E, Duschl A, Puntes V, Oostingh GJ. Shape matters: effects of silver nanospheres and wires on human alveolar epithelial cells. Part Fibre Toxicol. 2011;8:36.

Tsai CY, Lu SL, Hu CW, Yeh CS, Lee GB, Lei HY. Size-dependent attenuation of TLR9 signaling by gold nanoparticles in macrophages. J Immunol. 2012;188(1):68-76.

Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano. 2008;2(10):2121-34.

Zhang H, Dunphy DR, Jiang X, Meng H, Sun B, Tarn D, Xue M, Wang X, Lin S, Ji Z, Li R, Garcia FL, Yang J, Kirk ML, Xia T, Zink JI, Nel A, Brinker CJ. Processing pathway dependence of amorphous silica nanoparticle toxicity: colloidal vs pyrolytic. J Am Chem Soc. 2012;134(38):15790-804.

1.5. Characterization of the Bio-Corona and Role in Toxicity

Nanoparticles do not present a ‘naked’ surface in a biological system; instead, particles adsorb biomolecules including proteins and lipids leading to the formation of a so-called *bio-corona* (Monopoli et al., 2012). Though the dynamic adsorption and desorption of proteins onto surfaces has been known for several years (Vroman, 1962), this has been implicated in modulating the functionality and toxicity of ENMs during the past decade, and the bio-corona is now believed to be an important determinant of the biological effects of ENMs. Considerable research efforts have been devoted to this topic since the pioneering work of Prof. Sara Linse at Lund University in collaboration with Prof. Kenneth Dawson at University College Dublin (Cedervall et al., 2007), and we now know that size and surface properties determine the composition of the protein corona (Lundqvist et al., 2008). There is also evidence for the formation of a lipid corona on nanoparticles (Hellstrand et al., 2009; Kapralov et al., 2012. Furthermore, proteomics-based profiling studies have shown that the bio-corona ‘fingerprint’ dictates cellular interactions (Walkey et al., 2014). In fact, the authors could show that a multivariate model that uses the corona fingerprint was able to predict cell association far more accurately than a model that uses material intrinsic parameters such as nanoparticle size, aggregation state, and surface charge. Taken together, it appears that the combination of material intrinsic properties ('synthetic identity') and context-dependent properties determined, in part, by the bio-corona ('biological identity') determines the interactions of ENMs with cells and tissues and the subsequent outcomes (Fadeel et al., 2013; Pietroiusti et al., 2013) (Figure 5). Importantly, while a majority of the early work on the bio-corona focused on the formation of a protein corona on nanoparticles immersed in human plasma or fetal bovine serum (FBS), more recent studies have begun to address the potential role of the protein corona using *in vivo* models (Hadjidemetriou et al., 2015; Bertrand et al., 2017). The formation of a bio-corona naturally has implications for biomedical applications of nanoparticles for drug delivery and/or imaging (Schöttler et al., 2016; Tonigold et al.,2018). Indeed, some authors have proposed that the adsorption of serum proteins may mask targeting ligands on the surface of the nanoparticles (Salvati et al., 2013). On the other hand, targeted uptake of nanoparticles in cancer cells can also occur in the presence of serum (Krais et al., 2014) (and see: Schöttler et al., 2016).

The composition of the bio-corona largely depends on the biological milieu. Hence, when nanoparticles are present inside the alveolar region of the lungs they are coated with a bio-corona rich in both lipids and

proteins of a different composition as compared to the blood stream (Wohlleben et al., 2016; Tenzer et al., 2013). Less is known regarding the bio-corona in other compartments, such as the gastrointestinal tract. However, the route of exposure will determine the composition of the bio-corona.

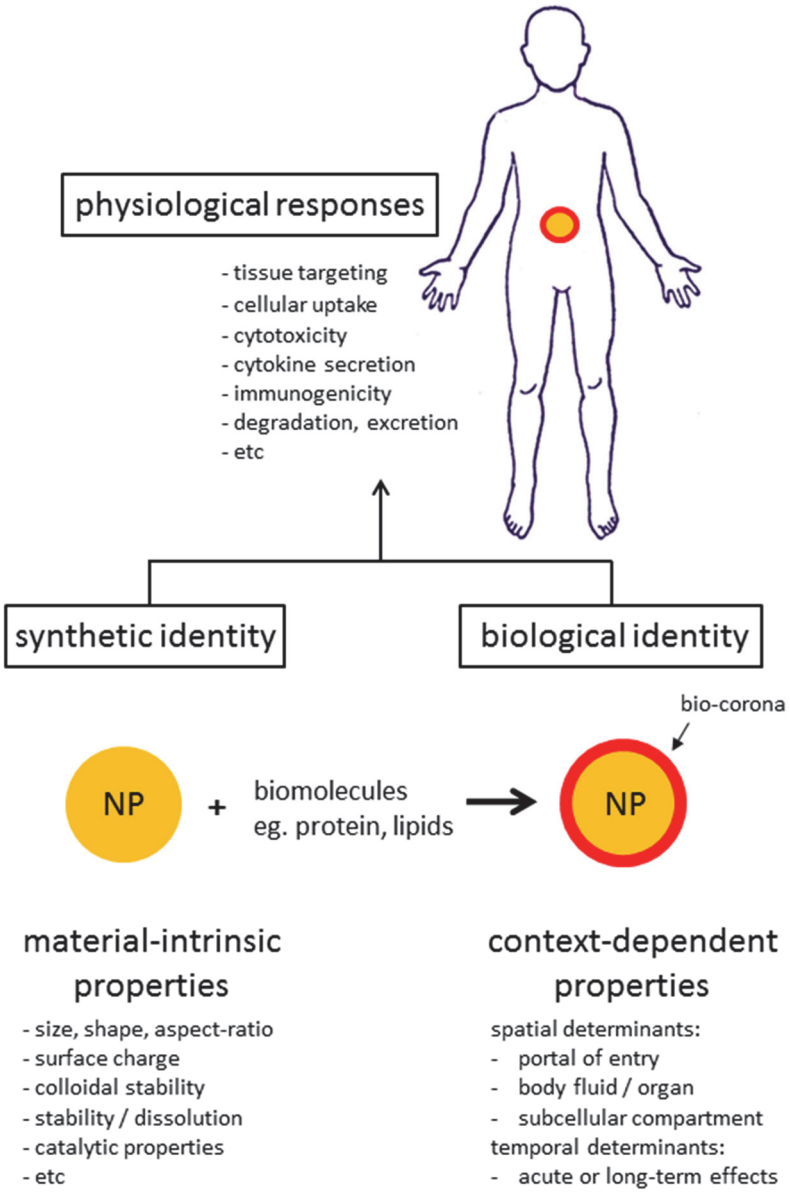


Figure 5. Schematic view of the 'synthetic' identity of ENMs determined by material-intrinsic properties and the 'biological' identity manifested in a living system and shaped by the adsorption of biomolecules on the particle surface. Adapted from: Fadeel et al., 2013.

From the perspective of nanosafety, the bio-corona has been found to be involved in controlling the toxicity of nanoparticles. Ten years ago, Dutta et al. (2007) investigated the importance of proteins adsorbed onto the

surface of single-walled carbon nanotubes (SWCNTs) *versus* amorphous silica nanoparticles in terms of uptake or toxicity in a murine macrophage-like cell model. Albumin was identified as the major fetal bovine or human serum/plasma protein adsorbed onto SWCNTs, while the profile of proteins adsorbed onto amorphous silica particles was qualitatively different. Overall, the results suggested an important role for adsorbed proteins in modulating the uptake and toxicity of SWCNTs and amorphous silica. Deng et al. (2011) showed that negatively charged poly(acrylic acid)-conjugated gold nanoparticles bind to and induce unfolding of fibrinogen, which promotes interaction with integrin receptors with activation of the NF-κB signaling pathway, resulting in the release of inflammatory cytokines. In a subsequent study, the authors examined plasma protein binding to gold nanoparticles with different surface charge (Deng et al., 2013). Fibrinogen bound with high affinity to both of the charged nanoparticles. However, only the negatively charged nanoparticles induced cytokine release from THP-1 cells. Thus, while common proteins can bind to different nanoparticles, the biological outcome may not always be the same. In another study, coating of carbon soot powder or TiO_2 and SiO_2 nanoparticles with fibrinogen was found to increase cytotoxicity and inflammatory responses in an alveolar macrophage cell line (Marucco et al., 2016). This is in contrast to other studies showing that coating with FBS passivates nanoparticles and reduces their toxicity (Maiorano et al., 2016). However, it is important to note that fibrinogen (and complement factors) are abundant in plasma, but absent in serum. Vogt et al. (2015) determined the composition of the human plasma protein corona on silica-coated *versus* dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs) (used for MRI) using mass spectrometry-based proteomics approaches. Notably, gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed distinct protein corona compositions for the two different SPIONs. The viability of primary human monocyte-derived macrophages was influenced by the protein corona on silica-coated SPIONs, and the protein corona promoted cellular uptake of silica-coated SPIONs, but did not affect internalization of dextran-coated SPIONs. No pro-inflammatory cytokine release was observed (Vogt et al., 2015). Tenzer et al. (2013) found that corona formation on silica nanoparticles affects haemolysis, thrombocyte activation, and endothelial cell death at an early exposure time. However, despite the ever increasing number of publications on the bio-corona, there is still no consensus or easy answer to the question whether the bio-corona may promote or mitigate toxicity, or whether or not the bio-corona will prevent or facilitate cellular uptake of nanomaterials, and we must conclude at this point that the bio-corona needs to be taken into account, but its impact on the

biological behavior of ENMs should be evaluated on a case-by-case basis. Furthermore, the *in vivo* relevance requires further exploration, and the bio-corona in different compartments in the body should also be studied in more detail; at present, there is a propensity of studies on bio-corona formation in the blood, and less on the bio-corona formation that may take place in other organs in the body. Methods for qualitative analysis of the bio-corona including high-resolution mass-spectroscopy techniques have been applied, but these need to be complemented with quantitative measurements of the bio-corona. Furthermore, it may also be important to consider single nanoparticles one by one, instead of looking at several billion particles simultaneously as has been the case until now, according to a recent study from Chalmers Technical University (Alekseeva et al., 2017). This elegant study opens a host of new questions as it suggests that nanoparticles should be considered as 'individuals' – this may affect our understanding of bio-corona formation and of the biological or toxicological interactions of ENMs, and seems to indicate that traditional analytical methods may not be sufficient. Finally, it remains to be understood if and how the bio-corona should be taken into consideration in risk assessment of the health impact of ENMs.

"it remains to be understood if and how the bio-corona should be taken into consideration in risk assessment"

References

Alekseeva S, Fanta ABDS, Iandolo B, Antosiewicz TJ, Nugroho FAA, Wagner JB, Burrows A, Zhdanov VP, Langhammer C. Grain boundary mediated hydriding phase transformations in individual polycrystalline metal nanoparticles. Nat Commun. 2017;8(1):1084.

Bertrand N, Grenier P, Mahmoudi M, Lima EM, Appel EA, Dormont F, Lim JM, Karnik R, Langer R, Farokhzad OC. Mechanistic understanding of in vivo protein corona formation on polymeric nanoparticles and impact on pharmacokinetics. Nat Commun. 2017;8(1):777.

Cedervall T, Lynch I, Lindman S, Berggård T, Thulin E, Nilsson H, Dawson KA, Linse S. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci U S A. 2007;104(7):2050-5.

Deng ZJ, Liang M, Monteiro M, Toth I, Minchin RF. Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. Nat Nanotechnol. 2011;6(1):39-44.

Deng ZJ, Liang M, Toth I, Monteiro M, Minchin RF. Plasma protein binding of positively and negatively charged polymer-coated gold nanoparticles elicits different biological responses. Nanotoxicology. 2013;7(3):314-22.

Dutta D, Sundaram SK, Teeguarden JG, Riley BJ, Fifield LS, Jacobs JM, Addleman SR, Kaysen GA, Moudgil BM, Weber TJ. Adsorbed proteins influence the

biological activity and molecular targeting of nanomaterials. Toxicol Sci. 2007;100(1):303-15.
Fadeel B, Feliu N, Vogt C, Abdelmonem AM, Parak WJ. Bridge over troubled waters: understanding the synthetic and biological identities of engineered nanomaterials. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2013;5(2):111-29.
Hadjidemetriou M, Al-Ahmady Z, Mazza M, Collins RF, Dawson K, Kostarelos K. In vivo biomolecule corona around blood-circulating, clinically used and antibody-targeted lipid bilayer nanoscale vesicles. ACS Nano. 2015;9(8):8142-56.
Hellstrand E, Lynch I, Andersson A, Drakenberg T, Dahlbäck B, Dawson KA, Linse S, Cedervall T. Complete high-density lipoproteins in nanoparticle corona. FEBS J. 2009;276(12):3372-81.
Kapralov AA, Feng WH, Amoscato AA, Yanamala N, Balasubramanian K, Winnica DE, Kisin ER, Kotchey GP, Gou P, Sparvero LJ, Ray P, Mallampalli RK, Klein-Seetharaman J, Fadeel B, Star A, Shvedova AA, Kagan VE. Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration. ACS Nano. 2012;6(5):4147-56.
Krais A, Wortmann L, Hermanns L, Feliu N, Vahter M, Stucky S, Mathur S, Fadeel B. Targeted uptake of folic acid-functionalized iron oxide nanoparticles by ovarian cancer cells in the presence but not in the absence of serum. Nanomedicine. 2014;10(7):1421-31.
Lundqvist M, Augustsson C, Lilja M, Lundkvist K, Dahlbäck B, Linse S, Cedervall T. The nanoparticle protein corona formed in human blood or human blood fractions. PLoS One. 2017;12(4):e0175871.
Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci U S A. 2008;105(38):14265-70.
Maiorano G, Sabella S, Sorce B, Brunetti V, Malvindi MA, Cingolani R, Pompa PP. Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response. ACS Nano. 2010;4(12):7481-91.
Marucco A, Gazzano E, Ghigo D, Enrico E, Fenoglio I. Fibrinogen enhances the inflammatory response of alveolar macrophages to TiO2, SiO2 and carbon nanomaterials. Nanotoxicology. 2016;10(1):1-9.
Monopoli MP, Åberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. Nat Nanotechnol. 2012;7(12):779-86.
Pietroiusti A, Campagnolo L, Fadeel B. Interactions of engineered nanoparticles with organs protected by internal biological barriers. Small. 2013;9(9-10):1557-72.
Salvati A, Pitek AS, Monopoli MP, Prapainop K, Bombelli FB, Hristov DR, Kelly PM, Åberg C, Mahon E, Dawson KA. Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface. Nat Nanotechnol. 2013;8(2):137-43.
Schöttler S, Becker G, Winzen S, Steinbach T, Mohr K, Landfester K, Mailänder V, Wurm FR. Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. Nat Nanotechnol. 2016;11(4):372-7.
Schöttler S, Landfester K, Mailänder V. Controlling the stealth effect of nanocarriers through understanding the protein corona. Angew Chem Int Ed Engl. 2016;55(31):8806-15.

Tenzer S, Docter D, Kuharev J, Musyanovych A, Fetz V, Hecht R, Schlenk F, Fischer D, Kiouptsi K, Reinhardt C, Landfester K, Schild H, Maskos M, Knauer SK, Stauber RH. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat Nanotechnol. 2013;8(10):772-81.

Tonigold M, Simon J, Estupiñán D, Kokkinopoulou M, Reinholz J, Kintzel U, Kaltbeitzel A, Renz P, Domogalla MP, Steinbrink K, Lieberwirth I, Crespy D, Landfester K, Mailänder V. Pre-adsorption of antibodies enables targeting of nanocarriers despite a biomolecular corona. Nat Nanotechnol. 2018 Jun 18. [Epub ahead of print].

Vogt C, Pernemalm M, Kohonen P, Laurent S, Hultenby K, Vahter M, Lehtiö J, Toprak MS, Fadeel B. Proteomics analysis reveals distinct corona composition on magnetic nanoparticles with different surface coatings: implications for interactions with primary human macrophages. PLoS One. 2015;10(10):e0129008.

Vroman L. Effect of absorbed proteins on the wettability of hydrophilic and hydrophobic solids. Nature. 1962;196:476-7.

Walkey CD, Olsen JB, Song F, Liu R, Guo H, Olsen DW, Cohen Y, Emili A, Chan WC. Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano. 2014;8(3):2439-55.

Wohlleben W, et al. Influence of agglomeration and specific lung lining lipid/protein interaction on short-term inhalation toxicity. Nanotoxicology. 2016;10(7):970-80.

2. Exposure Assessment

2.1. Occupational and Consumer Exposure to Nanomaterials

ENMs are increasingly used in applications such as consumer products, mainly personal care products and clothing, and in medical products for imaging and drug delivery systems. The emerging uses and the speed at which nanomaterial products are developed opens up for hazardous human exposure to several different nanomaterials resulting in the challenging task to assess the risks for human health. However, we must not forget that certain nanomaterials such as titanium dioxide (TiO_2) and carbon black pigments have been used for a long time without evidence of overwhelming health effects from exposure. On the other hand, nanoparticles generated in processes such as milling and grinding of ores, have proven to give rise to unintentional exposure with serious illnesses such as silicosis. Here, an overview is provided of the current knowledge on occupational and consumer exposure to ENMs, the regulatory status for ENMs in the occupational setting, and exposure assessment methods such as direct measurements and tiered approaches for risk assessment.

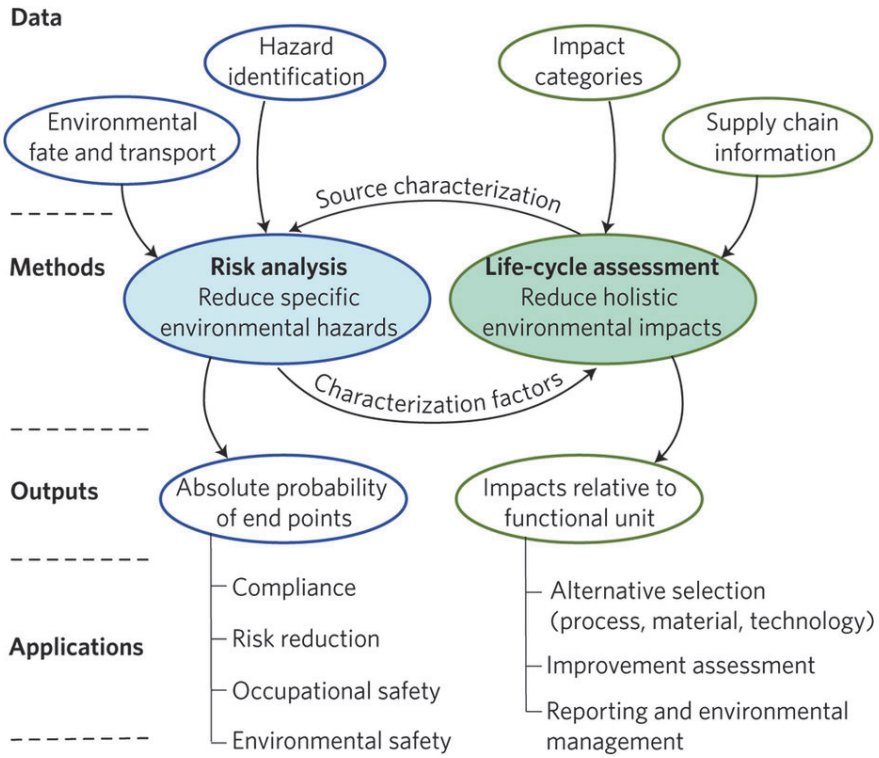


Figure 6. Schematic representation of the connections between risk assessment and life cycle assessment in the context of nanotechnology applications. From: Linkov et al., 2017.

Occupational exposure

In 2009, the European Agency for Safety and Health at Work (EU-OSHA) identified the sectors of construction, health care, energy conversion and use, automobile and aerospace industry, chemical industry and electronics and communication as the fields of industry where most workers are at risk of being exposed to nanomaterials (EU-OSHA, 2009). In particular, occupational exposure to engineered nano-objects and their agglomerates and aggregates (NOAA) has attracted much attention. It is important to stress that the form of exposure may vary throughout the life cycle of ENMs, correlating to production, handling of nano-powder, dispersion from ready-to-use products, and at end-of-life handling (Schneider *et al.*, 2011). It is worth noting in this context that two analytic perspectives dominate environmental policy and decision-making namely risk analysis and life cycle analysis (LCA). The former focuses on management of a toxicological hazard in a specific exposure scenario, while LCA seeks to develop a holistic understanding of multiple impacts across the life cycle of a product or service (Linkov et al., 2017) (Figure 6).

The major route of exposure to ENMs in the occupational setting is *inhalation*. During the last decade, many studies have reported methods to measure and assess the exposure to airborne ENMs. Recently, two review papers on ENMs and occupational exposures have been published (Debia et al., 2016; Ding et al., 2017). Ding et al. focus on the type and quantities of ENMs and which processes that leads to release of ENMs into the work air during different production stages. It was shown that the majority of studies performed between the years 2004-2016, could be grouped into three categories based on the aim of the study: (1) characterisation, (2) sampling strategy, or (3) contextual information (Ding, et al., 2017). Furthermore, the authors identify that high-energy processes such as spraying, synthesis and different types of machining activities, generate small particles at large numbers. Processes such as cleaning, industrial packaging and laboratory handling of ENMs more often resulted in large agglomerates. Moreover, the most common ENMs in the reviewed studies were shown to belong to carbon-based nanomaterials, metals and metal oxides nanoparticles. The authors found that individual exposure by personal air monitoring in the breathing zone is rarely measured. More commonly, point sources are assessed and at best a comment on descriptive factors such as ventilation rate or airflow rates in the room is available. This makes it difficult to estimate the particle release rates from the processes, which is important to limit exposure to the workers (Ding et al. 2017).

Debia et al. performed a systematic review of 6403 publications between 2000 and 2015, of which 50 studies describing 306 exposure situations in 72 workplaces were eligible for inclusion (27 industrial-scale plants and 45 research or pilot-scale units) (Debia et al., 2016). The quality of evidence for the conclusion statements was rated as low, moderate, or high depending on the number of confirmed exposure situations, the strength of the exposure assessment, and the consistency of the results. Regarding the potential of exposure in the workplace, the authors found high-quality evidence for multi-walled carbon nanotubes (CNTs), single-walled CNTs, carbon nanofibers, aluminium oxide, titanium dioxide, and silver nanoparticles; moderate-quality evidence for non-classified CNTs, nanoclays, and iron and silicon dioxide nanoparticles; low-quality evidence for fullerenes, double-walled CNTs, and zinc oxide nanoparticle; and no evidence for cerium oxide nanoparticles. Furthermore, they found high-quality evidence that exposure is most frequently due to handling tasks, that workers are mostly exposed to micro-sized agglomerates of nanoparticles, and that engineering controls considerably reduce worker exposure.

“Exposure is most frequently due to handling tasks, and engineering controls considerably reduce worker exposure.”

The reviews by Ding et al. (2017) and Debia et al. (2016) point to the fact that prevention and exposure assessment in occupational settings has to be improved and harmonized, which may be a challenge due to the versatility in different usages and behaviour of nanoparticles in current and future use. In 2015, the Organization for Economic Co-operation and Development (OECD) published the report “Harmonized tiered approach to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates” (OECD, 2015) in which a 3-step tiered approach is provided to evaluate the exposure (1) from the gathering of information on materials handled, the processes used and relevant workplace activities, (2) from using a basic exposure assessment, and (3) by obtaining all information on airborne nano-objects in the workplace. The focus of tier 3, is to measure the exposure of NOAAs in the workplace air. It is important to note that the OECD document does not include a health-based strategy to assess toxicity; hence, it cannot be used as a strategy for risk assessment. To reduce occupational exposure of hazardous substances, an occupational exposure limit value (OEL) may be set. Within the EU, this work is

performed by the Scientific Committee on Occupational Exposure Limits (SCOEL) with “the objective in establishing OELs is to set limits for exposure via the airborne route such that exposure, even when repeated on a regular basis throughout a working life, will not lead to adverse effects on the health of exposed persons and/or their progeny at any time (as far as can be predicted from the contemporary state of knowledge)” (SCOEL, 2013). The OEL may be either health-based, i.e., derived from knowledge of toxicological studies to establish a threshold concentration below which no adverse health effect is likely to occur, or risk-based for some adverse effects where it is not possible to define a threshold i.e. carcinogenicity, genotoxicity or respiratory sensitisation. Currently there is a lack of health based regulatory OELs for NOAAs (OECD, 2015), but several agencies and national authorities are evaluating the possibility of developing OELs. Different strategies have been discussed to come to terms with the current lack of data. The key scientific factors that impact the occupational exposure to airborne NOAAs have been summarised by the OECD: (1) lack of health based regulatory OELs, (2) lack of appropriate exposure metrics and, (3) behavior of airborne NOAA. The EU funded project SCAFFOLD, focusing on the use and exposure of ENMs within the construction sector, recently published a report on the formulation of OELs for inhalation and dermal exposure that provides a basis for potential OELs for several common ENMs, i.e., amorphous silicon dioxide, titanium dioxide, carbon nanofibers, nanocellulose, nanoclays, and general low-toxicity dust (SCAFFOLD, 2014). SCAFFOLD proposed that the substance-specific limit values proposed previously by the National Institute for Occupational Safety and Health (NIOSH) (TiO_2), the German Research Foundation (DGF) (SiO_2, as amorphous silica or silica fume) and NIOSH (carbon nanofibers) can be used for setting OELs. They suggested an 8 h OEL for the respirable fraction of 0.3 mg/m^3 (SiO_2), and 0.1 mg/m^3 (TiO_2) and 0.01 fibers/cm^3 for carbon nanofibers and nanocellulose based on the precautionary principle. No value could be set for nanoclays. For general low-toxicity dust, which comprises of a mixture of dust in the construction industry, the suggestion is 0.3 mg/m^3 for the respirable fraction (SCAFFOLD, 2014). In a recent systematic review of occupational exposure limits to manufactured nanomaterials (MNM), Mihalache et al. (2017) found a total of 20 studies of high relevance to the topic. In these studies, 56 OELs were proposed by different organisational bodies (Mihalache et al., 2017). Suggestions were to use generic level OELs for all MNM or to group MNM into specific groups and apply a generic OEL for the groups. Some studies also suggested to use specific level OELs for specific nanomaterials, much like the existing OELs for other compounds. Looking at the variation of the suggested OELs for different groups of MNMs the authors found values ranging by a factor of 30 to 50

for carbon nanotubes and a factor of 100 to 300 for metals. This clearly indicates that there is a need for a more structured approach to setting OELS for MNMs and also show the difficulties that lie ahead of regulatory bodies before consensus can be reached on how to proceed with setting OELs.

The lack of health based regulatory OELs opens for risk assessment by a combination of quantitative and qualitative assessments to determine whether exposure to NOAA occurs or not. However, a purely quantitative assessment may not be relevant for workers' exposure. Another way forward would be to apply safety assessment factors for already established OELs for larger particles to assess the risk of NOAAs (OECD, 2015).

In the International Organization for Standardization (ISO) document "*Nanotechnologies – Overview of available frameworks for the development of occupational exposure limits and bands for nano-objects and their aggregates and agglomerates (NOAAs)"* (ISO, 2016), ISO acknowledges the need for OELs, but states that there is still too limited data to set OELs for NOAAs. If an OEL cannot be set, an occupational exposure band (OEB) may instead be used. In their report, ISO states that few OELs and OEBs have been developed for specific NOAAs, and that none of these have been "formally regulated by a government agency". In summary, several agencies and international bodies as well as national institutes are discussing on how to best assess the risks of occupational exposure to NOAAs. Some suggestions for actual limit values have been proposed by NIOSH, the British Standard Institute, Institute for Occupational Safety and Health of the German Social Accident Insurance, as well as different large projects like the EU-funded project, SCAFFOLD. At present, in our view, the biggest problem with non-existing OELs is that few measurements are performed because there is no limit value to compare the result with and the situation thus resembles a catch 22 sitation.

Consumer exposure

There is currently no legislation limiting ENMs in consumer products, and no specific legislation stating that consumers shall be informed about ENMs in consumer products, which make it difficult to estimate how many consumer products that do contain ENMs. However, the EU Cosmetics Regulation provides the only exception, since it states that the word "nano" must be clearly labelled on products containing ENMs. In 2009, EU-OSHA

estimated that some 400 products containing ENMs were available for the consumer market (EU-OSHA, 2009). This number has increased to 1800 products in 2014 (Park et al., 2017). To obtain a better overview of the extent to which consumer products contain ENMs, several attempts have been made to establish registers in different countries in order to collect information about ENM-containing products; The Consumer Product Inventory (CPI) mainly focuses on the North American and Asian market. Within Europe the register by European Association for co-ordination of Consumer Representation in Standardisation (ANEC)/The European Consumer Organisation (BEUC) focuses on silver nanoparticle-containing products. These registers are not currently up to date; ANEC/BEUC has not been updated since 2013, the CPI is updated annually (Hansen et al., 2016). The Nanodatabase was established by the EU-funded project ENVNANO (Hansen et al., 2016). It is a database mainly containing consumer products for the European market that is updated weekly. Currently, the Nanodatabase contains 2231 products of which the majority contain silver or titanium dioxide nanoparticles, mostly found within the category of “personal care” and “clothing” products (Hansen et al., 2016).

As opposed to occupational exposure, where inhalation is the greatest concern, it is clear that consumers are mostly exposed to ENMs through the *dermal route* since personal care products and clothing constitutes typical consumer products containing ENMs. One exception where exposure also may occur by inhalation is when the product is packed in a spray bottle. Several studies have evaluated the effect inhalation exposure to ENMs in spray products (Chen et al., 2010; Losert et al., 2014; Park, et al., 2017). Losert et al. reviewed the current knowledge on how to measure spray aerosols, nanoparticle characterisation and exposure modelling and came to the conclusion that the existing studies used too different experimental set-ups and too few products for a general conclusion to be drawn regarding exposure values (Losert et al., 2014). In another recent study, the aerosol from different spray containers with propellant spray nozzles and pump spray nozzles respectively, was compared. The result showed that the propellant spray nozzle gave rise to higher particle concentrations in the nano-range as compared to the pump spray nozzle, showing that not only the spray bottle but also the nozzle may affect the potential exposure through inhalation (Park et al., 2017).

Sunscreen protection

Dermal exposure to consumer products containing ENMs, mostly TiO_2 or ZnO, has mainly been studied for sunscreens and cosmetics. Numerous studies on TiO_2 or ZnO nanoparticles have been performed to evaluate the potential risks associated with the use of these materials as physical filters in topical products. Dermal toxicity to nanoparticles is discussed below in the section on Hazard Assessment. A summary can be found in reports from the EU Scientific Committee on Consumer Safety (SCCS), and a recent review by the Australian Government, Department of Health, Therapeutic Goods Administration (TGA, 2016/2017) (Chaudhry, 2015; Scientific Comittee on Consumer Safety, 2012; Theraputic Goods Administration, 2016). The SCCS declared that the use of TiO_2 and ZnO nanoparticles up to 25% as a UV-filter in sunscreens can be considered "to not pose a risk of adverse effects in humans after dermal application". However, in light of sunscreens being packaged into different types of spray bottles, the SCCS amended its statement and does currently not recommend the use of TiO_2 and ZnO nanoparticles in "spray applications that could lead to exposure of the consumer's lungs" (SCCS, 2012). In other words, the use of nanoparticulate TiO_2 and ZnO as UV-filters in sunscreens was judged to not pose a problem when applied topically on the skin, but it should not be used in spray bottles that may generate an aerosol of nanoparticles that possibly can be inhaled. Similar to the SCCS, the Australian TGA came to the conclusion that topically applied sunscreens on human skin, irrespectively if it is damaged or not, should not pose a risk to humans (TGA, 2016). A large number of scientific studies report that these types of nanoparticles do not penetrate the outermost layers of the skin, but mainly stays in epidermis and hence particles do not reach the viable cell layers (see further the chapter on dermal toxicity) (Baroli et al., 2007; Jeong et al., 2010; Palmer et al., 2016). The TGA stated that neither TiO_2 nor ZnO nanoparticles are likely to cause harm when used as ingredients in sunscreens when applied topically to the skin and, furthermore, the benefits of skin protection outweigh the risks (TGA, 2016).

2.2. Methods for Exposure Assessment and Particle Detection

Air exposure

Several different measurement approaches can be used to measure nanoparticles to quantify exposure and every technique presents with its own advantages and disadvantages. The most common measurements are based on number of particles in the air, surface concentration/area of particles, and the traditional approach based on measurements of the mass of particles in the air. The latter approach is difficult since small

particles carry a very low mass and in for example industrial settings, it may be very difficult to separate the mass of nanoparticles from that of larger particles also present in the air. Concerning the number of particles in air, which seems an adequate way forward, the main disadvantage is again related to difficulties to distinguish ENMs from background particles, due to large variations in spatial and temporal distribution. The surface concentration measurements of ENMs are important, but this is also hampered by the inherent properties of nanoparticles to quickly form agglomerates (Hedmer et al., 2015; Karlsson et al., 2015; Nilsson et al., 2013).

Many studies where nanoparticle exposure was quantified use several different instruments that usually account for all three metrics. Commonly used instruments are the scanning mobility particle sizer (SMPS) alone or in a combination with a condensation particle counter (CPC). Both instruments will give information on real-time size selective number concentrations providing an aerosol size distribution. For the purpose of measuring only the amount of particles as numbers/cm^3 an aerodynamic particle sizer (APS) or different versions of the so-called P-Trak and DustTrak instruments are often used. In order to actually prove the presence of nanoparticles in air at a workplace, traditional filter sampling is often performed for the visualisation by electron microscopy (SEM/TEM). Such techniques coupled to energy dispersive X-ray spectroscopy (EDX) can also provide the chemical composition of particles. With a size-selective sampler (SSS) or a tapered element oscillating microbalance (TEOM) the mass of nanoparticles may also be measured (Karlsson et al., 2015; Nilsson et al., 2013). The aerosol mass spectrometer (AMS) constitutes a state-of-the-art instrument in that it enables the characterization of airborne particles simultaneously for size and chemical composition online. However, this is not a portable instrument, which reduce its applicability for example in field studies to characterise occupational exposure in different work places (Nilsson et al., 2013). When measurements are not practically feasible, exposure assessment can still be performed by using different types of control banding approaches. Computer software has been developed for nanomaterials to evaluate the risk of exposure in the absence of detailed exposure information such as NanoSafer, Stoffenmanager-Nano, CB NanoTool, and the Precautionary matrix (Sanchez Jimenez et al., 2016). The computational tools estimates hazard based on different parameters such as physico-chemical characteristics of nanomaterials and toxicity data of related micron-sized particles. There is little information on how well these tools predict the empirical data in an occupational exposure

scenario, but they may be an option for a risk assessment in case of limited data. A current review demonstrate the limitations of the existing software for risk assessment and conclude that they should be used with care, and only to the specific aim that the tools have been developed, i.e., specific activities such as powder handling (refer to: Sánches-Jiménez et al., 2016).

Skin exposure

Traditional skin exposure assessment is usually measured by using one of the following main collection methods: removal techniques, surrogate skin techniques (interception techniques) and fluorescent tracer techniques (Fenske, 1993). These techniques should be possible to use for monitoring skin exposure to NOAAs as well. One prerequisite for a good sampling method is that it does not disturb the size, agglomeration or shape of the NOAA's when sampling, nor should this happen during the subsequent analysis of the sample. In a study by Brouwer et al. (2016), the authors provided an overview of strengthss and weaknesses of the three methods mentioned above to characterize and quantify the amounts of NOAA on skin (Brouwer et al., 2016). In short, using the surrogate skin technique with tradition gloves or patches placed on the skin may not be optimal for NOAA since these may be disturbed when removing the item from the body. The suggestion was made to use double-sided electron microscopy grade adhesive carbon tape to sample NOAAs, followed by microscopy analysis using a scanning electron microscope (SEM). The major advantage of removal techniques is that they actually measure what is on the skin. Fluorescent techniques should be optimal to assess the distribution of NOAAs on skin, since it is an *in situ* method not disturbing the NOAA during the measurement; however, not all NOAAs are fluorescent. The authors concluded by stating that fundamental research is needed to develop novel skin sampling methods and subsequent imaging methods for monitoring skin exposure to NOAAs (Brouwer et al., 2016).

Concluding remarks

Although no standardized measuring techniques are available, and the hazard statements are hampered to some extent due to a lack of exposure data, the current evidence of occupational exposure assessments suggests that workers are exposed to ENMs, though mainly in the form of agglomerates. It also seems evident that by following a precautionary principle and by reducing exposure by encapsulation of processes generating ENMs, with proper ventilation and the use of personal

protective equipment, the potential for exposure to ENMs may be reduced. Such preventive actions seem to offer the best solution, until OELs may can set for ENMs. Control banding may also be a way forward, but further development of tools for risk assessment is required (see section on Risk Assessment). Concerning consumer exposure, much would be gained from exploring the possibility of legislations for labelling of products that contain ENMs. Presently, only the EU Cosmetic Regulation requires such labelling.

References

Baroli, B., Ennas, M. G., Loffredo, F., Isola, M., Pinna, R., and Lopez-Quintela, M. A. (2007). Penetration of metallic nanoparticles in human full-thickness skin. The Journal of investigative dermatology 127(7), 1701-12.

Brouwer, D. H., Spaan, S., Roff, M., Sleeuwenhoek, A., Tuinman, I., Goede, H., van Duuren-Stuurman, B., Filon, F. L., Bello, D., and Cherrie, J. W. (2016). Occupational dermal exposure to nanoparticles and nano-enabled products: Part 2, exploration of exposure processes and methods of assessment. International journal of hygiene and environmental health 219(6), 503-12.

Chaudhry, Q. (2015). Opinion of the scientific committee on consumer safety (SCCS) - Revision of the opinion on the safety of the use of titanium dioxide, nano form, in cosmetic prducts. Regulatory toxicology and pharmacology 73(2), 669-670.

Chen, B. T., Afshari, A., Stone, S., Jackson, M., Schwegler-Berry, D., Frazer, D. G., Castranova, V., and Thomas, T. A. (2010). Nanoparticles-containing spray can aerosol: characterization, exposure assessment, and generator design. Inhalation toxicology 22(13), 1072-82.

Debia, M., Bakhiyi, B., Ostiguy, C., Verbeek, J. H., Brouwer, D. H., and Murashov, V. (2016). A Systematic Review of Reported Exposure to Engineered Nanomaterials. The Annals of occupational hygiene 60(8), 916-35.

Ding, Y., Kuhlbusch, T. A., Van Tongeren, M., Jimenez, A. S., Tuinman, I., Chen, R., Alvarez, I. L., Mikolajczyk, U., Nickel, C., Meyer, J., et al. (2017). Airborne engineered nanomaterials in the workplace-a review of release and worker exposure during nanomaterial production and handling processes. Journal of hazardous materials 322(Pt A), 17-28.

EU-OSHA (2009). Workplace exposure to nanoparticles.

Fenske, R. (1993). Dermal exposure assessment techniques. The Annals of occupational hygiene 37(6), 687-706.

Hansen, S., Roverskov Heggelund, L., Besora, P., Mackevica, A., Boldrin, A., and Baun, A. (2016). Nanoproducts - what is actually available to European consumers? Environmental Science Nano 3, 169-180.

Hedmer, M., Ludvigsson, L., Isaxon, C., Nilsson, P. T., Skaug, V., Bohgard, M., Pagels, J. H., Messing, M. E., and Tinnerberg, H. (2015). Detection of Multi-walled Carbon Nanotubes and Carbon Nanodiscs on Workplace Surfaces at a Small-Scale Producer. The Annals of occupational hygiene 59(7), 836-52.

International Organisation for Standardization (2016). Nanotechnologies - Overview of available frameworks for the development of occuaptinal exposure limits and bands fro nano-objects and their aggregates and agglomerates (NOAAs).

Jeong, S. H., Kim, J. H., Yi, S. M., Lee, J. P., Kim, J. H., Sohn, K. H., Park, K. L., Kim, M. K., and Son, S. W. (2010). Assessment of penetration of quantum dots through in vitro and in vivo human skin using the human skin equivalent model and the tape stripping method. Biochemical and biophysical research communications 394(3), 612-5.

Karlsson, H., Toprak, M. S., and Fadeel, B. (2015). Toxicity of Metal and Metal Oxide Nanoparticles. In: *Handbook on the Toxicology of Metals*. (G. F. Nordberg, B. A. Fowler, and M. Nordberg, Eds.), pp. 75-112. Academic Press.

Linkov I, Trump BD, Wender BA, Seager TP, Kennedy AJ, Keisler JM. Integrate life-cycle assessment and risk analysis results, not methods. Nature Nanotechnology. 2017;12(8):740-743.

Losert, S., von Goetz, N., Bekker, C., Fransman, W., Wijnhoven, S. W., Delmaar, C., Hungerbuhler, K., and Ulrich, A. (2014). Human exposure to conventional and nanoparticle--containing sprays-a critical review. Environmental science & technology 48(10), 5366-78.

Mihalache, R., Verbeek, J., Graczyk, H., Murashov, V., and van Broekhuizen, P. (2017). Occupational exposure limits for manufactured nanomaterials, a systematic review. Nanotoxicology 11(1), 7-19.

Nilsson, P. T., Isaxon, C., Eriksson, A. C., Messing, M. E., Ludvigsson, L., Rissler, J., Hedmer, M., Tinnerberg, H., Gudmundsson, A., Deppert, K., et al. (2013). Nano-objects emitted during maintainance of common particle generators: direct chemical characterization with aerosol mass spectrometry and implications for risk assessments. J Nanopart res 15, 2052.

OECD (2015). Harmonized tiered approch to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces. . Safety of Manufactured Nanomaterials.

Palmer, B. C., and DeLouise, L. A. (2016). Nanoparticle-Enabled Transdermal Drug Delivery Systems for Enhanced Dose Control and Tissue Targeting. Molecules (Basel, Switzerland) 21(12).

Park, J., Ham, S., Jang, M., Lee, J., Kim, S., Kim, S., Lee, K., Park, D., Kwon, J., Kim, H., et al. (2017). Spatial-Temporal Dispersion of Aerosolized Nanoparticles During the Use of Consumer Spray Products and Estimates of Inhalation Exposure. Environmental science & technology. 51(13):7624-7638.

Sanchez Jimenez, A., Varet, J., Poland, C., Fern, G. J., Hankin, S. M., and van Tongeren, M. (2016). A comparison of control banding tools for nanomaterials. Journal of occupational and environmental hygiene 13(12), 936-949.

SCAFFOLD (2014). Formulating occupational exposure limits values (OELs) (Inahalation & Dermal).

Schneider, T., Brouwer, D., Koponen, I., Jensen, K., Fransman, W., Van Duuren-Stuurman, B., Van Tongeren, M., and Tielemans, E. (2011). Conceptual model for assessment of inhalation exposure to manufactured nanoparticles. Journal of Exposure Science and Environmental Epidemiology 21, 450-463.

Scientific Comittee on Consumer Safety (2012). OPINION for clarification of the meaning of the term "sprayable applications/products" for the nano forms of Carbon Blac CI 77266, Titanium dioxide and Zinc Oxide.

Scientific Committee on Occupational Exposure Limits (2013). Methodology for the Derivation of Occupational Exposure Limits.

Theraputic Goods Administration, T. (2016). Literature review on the safety of titanium dioxide and zinc oxide nanoparticles in sunscreens.

3. Biodistribution

3.1. Uptake and Biodistribution of Nanomaterials

To establish the relationship between external exposure and toxicity, information on how nanoparticles are absorbed, distributed, metabolized and excreted from the body over time, i.e., biokinetics, is essential (Johanson, 2014). Biokinetic data can be derived from *in vitro*, *ex vivo*, and *in vivo* studies, and the mechanisms underlying the biological interactions and transport across biological barriers can be explored (Braakhuis et al., 2015; Frohlich and Salar-Behzadi, 2014; Johanson and Carlander, 2016). In the next step, the data that is generated can be used to develop *in silico* models with the aim to correlate external exposure to target dose. The present section describes the current knowledge regarding dermal, oral and inhalation exposure to ENMs in relation to the biodistribution of ENMs.

Cell-based *in vitro* studies can be carried out to gain a basic understanding of how ENMs cross biological barriers and interact with a biological system. Simple systems, with monocultures grown in wells, have been used to provide information about mechanisms and kinetics of cellular uptake including the impact of NP properties, corona formation, dissolution and dose-metrics into consideration (DeLoid et al., 2015; Lesniak et al., 2012; Lesniak et al., 2013; Lunov et al., 2011; Misra et al., 2012; Teeguarden et al., 2007). The uptake kinetics can be assessed by sampling at different time points and measuring both intra- and extracellular concentrations. The cellular uptake mechanisms can be addressed by use of imaging techniques as Raman spectroscopy, and confocal and electron microscopy combined with inhibitors of various pathways of cellular uptake (Brandenberger et al., 2010; Canton and Battaglia 2012; Iversen et al., 2011). More advanced *in vitro* systems are also available, i.e., with multi-cell cultures grown on the apical and basolateral side of semipermeable filters in specialized inserts, so-called Transwells. These more advanced systems provide more biologically relevant models as the translocation kinetics over biological barrier can be investigated (Bachler et al., 2015; Braakhuis et al., 2015). One such *in vitro* system is the air-liquid interface (ALI) model, which simulates the epithelial lining of the tracheobronchial region of the human respiratory tract. Characterization of the translocation kinetics requires an intact cellular barrier (George et al., 2015; Srinivasan et al., 2015). In addition, *ex vivo* systems can be used to characterize transport of nanopartricles in the lung and across the gastrointestinal tract (Brun et al., 2014; Meiring et al., 2005).

To gather information about biokinetics in intact organisms, biodistribution studies are conducted, typically with rodents. A variety of exposure conditions and several types of nanoparticles have been evaluated (Johanson and Carlander 2016; Kermanizadeh et al., 2015). The biokinetics of nanoparticles are complex and influenced by several factors such as physicochemical properties (size, shape, surface charge, coatings, agglomeration, dissolution, etc), exposure conditions (exposure method, route, duration, frequency, pattern, dose, etc) and animal model (age, sex, diseases, and species). To link toxicity to biokinetics, detailed information and understanding of these factors is crucial. Biodistribution studies have demonstrated that nanoparticles do not easily cross biological barriers (Johanson and Carlander 2016; Kermanizadeh et al., 2015). To overcome these barriers and improve the systemic delivery (in nanomedicine), nanoparticles can be administered intravenously. Once absorbed into the systemic circulation, nanoparticles are to a large extent transported to and captured by phagocytic cells in organs belonging to the mononuclear phagocyte system wherein the nanoparticles reside for extended periods (Gustafson et al., 2015; Saba, 1970). Extensive efforts have been made to design nanoparticles that avoid phagocytic recognition, often by using different types of coatings (Mahmoudi et al., 2016; Owens and Peppas 2006). However, this task has been more challenging than expected, due to difficulties to understand and predict facts that influence agglomeration, corona formation, and dissolution (Bruinink et al., 2015; Docter et al., 2015; Utembe et al., 2015). Elimination from the body is generally slow for nanoparticles unless they are small enough (or become small by dissolution) to be cleared to urine (< 5nm) and feces (Choi et al., 2007; Iavicoli et al., 2016). If the absorption rate exceeds the clearance rate, repeated exposure leads to bioaccumulation. Characterization of the biokinetics involves sampling of multiple organs, tissues and body fluids at several time-points .Repeat dosing and long post exposure follow-up are essential to capture long term effects and bioaccumulation. Careful tissue sampling, preparation and analysis is important as there are many steps that may impact on the results (reviewed in: Johanson and Carlander 2016). Quantification of the concentration/amounts of nanoparticles in tissue samples can be performed using different techniques. Inductive coupled plasma mass spectrometry (ICP-MS) seems to be the most widely used analytical

“Biodistribution studies have demonstrated that nanoparticles do not easily cross biological barriers.”

method for inorganic nanoparticles and offers low detection limits, generally down to nanograms per gram. Other analytical approaches use radioactive or fluorescent labels for quantification, some of which can also provide real-time monitoring. Imaging techniques such as magnetic resonance imaging (MRI) and X-ray can be applied on certain nanoparticles, e.g., iron oxide and gold nanoparticles. To visualize nanoparticles at the cellular level in tissues and organs, microscopy techniques such as transmission electron microscopy (TEM) can be used. Elementary composition and imaging of individual cells can be captured by ion beam spectroscopy techniques such as microproton-induced X-ray emission (μPIXE) and micro-Rutherford backscattering (μRBS) spectroscopy. Progress in the development of analytical methods with improved sensitivity and real-time visualization will improve the ability to characterize the interaction of nanoparticles with tissues and fluids in the body.

Dermal exposure

The skin is the largest organ (15% of the body weight) with a surface area in adults of approximately 2 m^2. Intact skin provides a good barrier against uptake of nanoparticles. To reach the systemic circulation, the nanoparticles first have to penetrate the outermost layer of the epidermis, stratum corneum. Penetration may occur *via* hair follicles, sweat glands or intra/intercellular routes, where passage *via* hair follicles and sweet glands is the most probable route for nanoparticles (Filon et al., 2016). Intercellular routes are limited to small nanoparticles and intracellular routes are primarily accessible for chemical substances and ions (Filon et al., 2015; Filon et al., 2013; Midander et al., 2016). However, several factors such as anatomical site (skin thickness), skin humidity, temperature, barrier integrity, mechanical flexion, nanoparticle properties, contaminants, and dissolution of nanoparticles may increase the apparent uptake (Filon et al., 2015; Filon et al., 2011; Filon et al., 2016; Johanson and Rauma 2008; Tinkle et al., 2003). Numerous studies have examined the dermal penetration of various types of ENMs, mostly by using different microscopic imaging techniques. These studies have demonstrated that an overwhelming proportion of the topically applied nanomaterial stays on the surface or in the outermost layers of stratum corneum. Besides, almost all studies have failed to demonstrate penetration beyond the epidermis. One limitation of these studies is that they are qualitative rather than quantitative in nature, and detection limits are not available (Johanson and Carlander, 2016). A few studies have used sensitive analytical methods to measure absorption through skin *in vitro* with diffusion cells, mainly by using metal-containing nanoparticles and analysis by ICP-MS. The

amount that translocate human skin *in vitro* is mostly low, with reported ranges from 0.0007% (silver, 25 nm) to 0.5% (gold, 13 nm) per 24 h (Johanson and Carlander 2016). However, higher uptake has been measured, as for 15-nm gold where 6% was absorbed by rat skin after 24 h (Sonavane et al., 2008). Many factors besides the physicochemical properties of the nanoparticles themselves may influence the results, including the applied amount/concentration, agglomeration, the composition of donor and receptor media, and design of the diffusion cell. Two major factors that may contribute to falsely high absorption values should be mentioned: disruption of the skin barrier during preparation and, for metal nanomaterials, dissolution of metal prior to absorption. It is well known that metal and metal oxide once placed in biologic media, releases metal ions which have higher permeability compared to intact nanoparticles (Midander et al., 2007; Midander et al., 2016; Midander et al., 2007).

Oral expoure

Upon oral exposure, ingested nanoparticles first pass the oral cavity, then the stomach, followed by the small intestine and the large intestine before being excreted with feces. The different parts of the gastrointestinal tract differ widely with respect to morphological structure, surface area, epithelial mucus layer thickness, and residence time (Frohlich and Roblegg, 2016). Throughout the gastrointestinal system, nanoparticles interact with the local environment including ingested food and beverage (Cao et al., 2016). Proteins and biomolecules adhere to the particle surface forming a bio-corona, which in turn may affect the penetration of nanoparticles and nutrients through mucus and epithelia (Di Silvio et al. 2016) (Docter et al., 2015) (Cao et al., 2016; Dorier et al., 2015; Mahler et al., 2012). Increased absorption may also be a result of ion release, as the harsh environment in parts of the gastrointestinal system may accelerate dissolution of the nanoparticles (Wang et al., 2014). The largest surface area is found in the small intestine and it is from here the majority of the translocation into the systemic circulation presumably occurs, although this has not been thoroughly verified. Before the nanoparticles can reach the systemic circulation, they have to penetrate the mucus and overcome the epithelium layer (des Rieux et al., 2006). In similarity with the mucus in the lung, penetration increases with decreased size (< 1% of the total 10 nm), and neutral and hydrophilic particles are favorable (Frohlich and Roblegg ,2016; Lai et al., 2009). Nanoparticles trapped in the mucus are transported distally to feces because of the renewal of mucus. The cellular barrier dominates by enterocytes, but the uptake is assumed to occur *via* the less abundant M-cells that occupy approximately 1% of the total

surface area. However, as few studies have sampled lymph nodes or visualized translocation, the mechanism by which transport across the gut barrier is still poorly understood. The systemic uptake varies substantially for different types of nanoparticles, ranging from undetectable to several per cent of the administered dose (Johanson and Carlander, 2016). The translocation of nanoparticles across gastrointestinal barriers has been studied both *in vitro*, *ex vivo*, and *ex vivo*. Studies using cultured cells (Bouwmeester et al., 2011; Win and Feng 2005) and isolated rat intestine (Sinnecker et al. 2014; Sonavane et al. 2008) have shown variable penetration, ranging from undetectable levels to several percent, while *in vivo* studies report data suggesting high (> 10%) to low (< 0.1%) oral absorption (Geraets et al., 2014; Hillyer and Albrecht 2001; Jani et al., 1994; Park et al., 2009; Singh et al., 2013; Wang et al., 2007; Yang et al., 2013). Studies conducted with silver and zinc oxide nanoparticles are not useful to assess the absorption of nanoparticles, due to the dissolution and formation of metal ions in gastric juice. Several studies could not demonstrate any oral absorption, i.e., no increase in nanoparticle levels in tissues, while one study with gold nanoparticles showed 0.03-0.37% size- and charge-dependent uptake, with higher uptake for the smaller particles (Schleh et al., 2012). On the other hand, a single study on silica (Lee et al., 2014) suggested oral uptake of several per cent of the administered dose.

Inhalation

Inhalation of airborne nanoparticles (in particle and aerosol research more commonly known as ultrafine particles) results in various degrees of particle deposition in different parts of the respiratory tract. Next to anatomy (including changes in diseased lung and species differences) and breathing patterns (tidal volume, breathing frequency, breath-holding, inspiration/exhalation flow rates), particle size and geometry influences the deposition the most (Gustafsson et al., 2016; Hofmann, 2011; Yang et al., 2008). Models of lung deposition of nanoparticles are available for several species. Three basic deposition mechanisms are involved; diffusion, impaction and sedimentation (Hofmann, 2011; Yang et al., 2008). Other mechanisms involved are turbulence, interception, electrostatic deposition, and thermophoretic and diffusiophoretic forces, however, their impact on nanoparticle deposition are, thus far, not well-addressed in predictions of deposition. The total deposition fraction of inhaled particles in normal breathing humans varies substantially with particle size (Balashazy et al. 2003; Brain et al. 1976). Once deposited, the nanoparticles diffuse into the mucus and other lining fluids followed by instant interaction with ions, surfactants and proteins. As a result, the

deposited nanoparticles may agglomerate, degrade, migrate or become engulfed by lung cells (Schulze et al., 2011). The smaller the particle size, the more readily the nanoparticles disperse and transport through the fluids. The mucus that coats the epithelium in the tracheobronchial region consists of 90-98% water and has a net positive charge (Lai et al., 2009). Neutral and hydrophilic nanoparticles penetrate the mucus more efficiently than charged ones, as positively charged nanoparticles are trapped in the mucus while negatively charged and lipophilic nanoparticles are repelled (Gustafsson et al., 2016). Particles that do not penetrate the mucus will be cleared by the mucociliary escalator. The surfactant that covers the epithelium in the alveolar region has both hydrophobic and hydrophilic properties, making the interaction between the surfactant and the nanoparticles complex and dependent on size, surface properties and shape.

Nanoparticles deposited in the lung can be cleared *via* several routes. Coughing, sneezing and swallowing, removes the particles in the upper respiratory tract, and to some extent also uptake into the brain via axons in olfactory nerves (DeLorenzo, 1970; Elder et al., 2006).The mucociliary escalator in the tracheobronchial region transports the particles to the larynx where they are swallowed. Alveolar macrophages may capture nanoparticles and migrate to the tracheobronchial region for subsequent mucociliary clearance or transport to mediastinal lymph nodes. Studies in rats have shown that with increased lung burden, transport to lymph nodes increases wheeras the mucociliary clearance from the pulmonary region decreases (Keller et al., 2014; Tran et al., 2000) (Morrow, 1988; Morrow et al., 1996). Impaired transport of particles *via* macrophages to the mucociliar escalator has been noted in rats exposed to high doses of poorly soluble particles, so called ‘particle overload’ (Morrow, 1988; Morrow et al., 1996). It is debated whether the overload phenomenon holds also for nanoparticles. It is also debated which dose-metric (total surface area or volume) drives the behavior and how to extrapolate from rodents to humans (Borm et al., 2015; Morfeld et al., 2015; Pauluhn, 2014).

Several animal studies report a small albeit significant increase in systemic levels of a main constituent after inhalation exposure to nanoparticles, suggesting that the nanoparticles are absorbed to a low but measurable degree *via* the respiratory route (Kermanizadeh et al. 2015). The systemic uptake of nanoparticles differs considerably between studies and it is difficult to judge if this variation is due to methodological issues (such as experimental design, exposure system, sampled organs, sampling time and species and strain differences), physicochemical properties (such as size, shape, degree of agglomeration and zeta potential) or simply reflects

method error. In addition, most studies have been carried out with metal or metal oxide nanoparticles and a major concern is that of dissolution and subsequent uptake of ions, not nanoparticles. However, most studies have addressed and seemingly controlled for this aspect. Another concern relates to repeated whole-body exposure in exposure chambers. In these experiments, there is a high risk of contamination or secondary exposure in animals caused by licking the fur. Nevertheless, the data suggest that the total systemic uptake, excluding mucosal clearance to the GI-tract of larger nanoparticles, is generally below 1% for the bigger (80 nm) particles and up to a few percent for the smaller ones (Johanson and Carlander 2016).

3.2. Physiologically Based Pharmacokinetic Modeling

Modeling biodistribution data aim to provide tools, such as physiologically based pharmacokinetic (PBPK) models, to correlate external exposure to target dose. Using mathematical descriptions of time courses for absorption, distribution, metabolization and excretion processes, the models calculates the biokinetics of nanoparticles administered *via* different routes of exposure (Johanson, 2014). PBPK models are used in risk assessment to extrapolate from high dose to low dose, from animals to humans and between exposure routes, to predict the target dose for different exposure scenarios, to estimate the toxicokinetic and target dose variability in a population, and to describe the relation between exposure and biomarker level. Another valuable aspect of PBPK models is their ability to generate hypotheses, aid in the design of biodistribution studies, and identify additional research needs. PBPK models use anatomical and physiological features, such as the structure of the circulatory system, organ and tissue volumes, and tissue blood flows, to calculate the mass flow of substances within organs and tissues in a time-dependent manner. Although PBPK models demonstrate good capability to predict kinetics of small molecules, their applicability to nanoparticles is challenging because of their complex biokinetics (Johanson and Carlander 2016; Li et al., 2017).

To make PBPK models useful, implementation of nanoparticle specific model structures and parameters is essential. So far, a variety of PBPK approaches have been used. To make PBPK models more general and useful for extrapolations between *in vitro* and *in vivo* situations as well as between types of nanoparticles, species, and exposure conditions, the models need to become more physiologically and physically relevant and include humans. This requires mechanistic understanding and input

values to model parameters. A promising step forward is the combined use of *in silico* tools (QSAR, QIVIVE, PBPK) with systematically designed *in vitro* and *in vivo* studies using well-characterized particles. However, data from repeated exposure and long-term exposure studies are lacking.

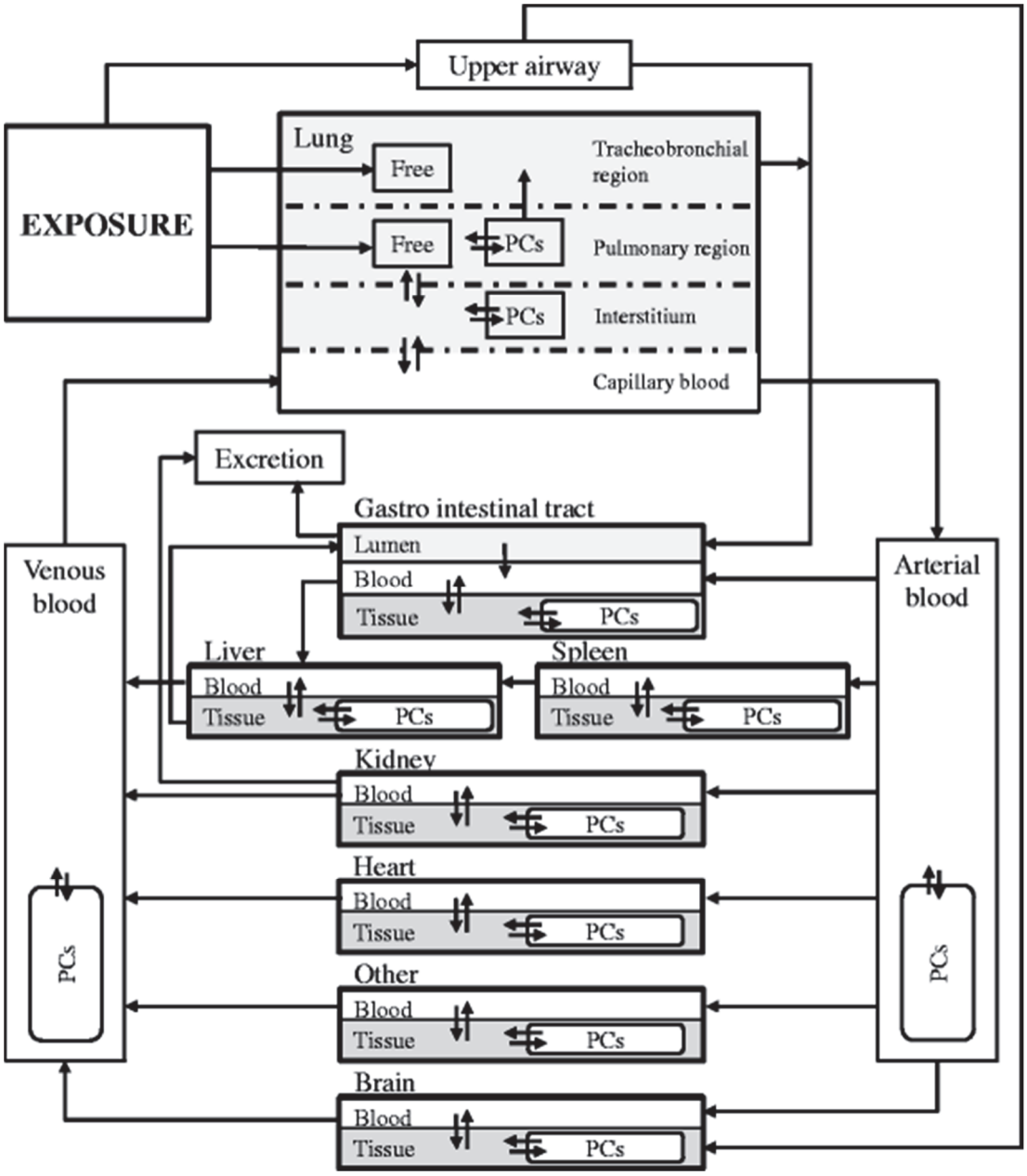


Figure 7. Schematic representation of a PBPK model developed for inhalation of nanoceria with sub-compartments of phagocytizing cells (PCs) in each tissue. From: Li et al. 2016.

Important processes known to affect the biokinetics of nanoparticles and that require further consideration in PBPK modeling of nanoparticles include bio-corona formation, agglomeration/aggregation, interaction with

and transport through cells, lymphatic transport, and nanoparticle dissolution. Nanoparticles in contact with a biological system tend to agglomerate and, at the same time, form a corona by attracting proteins to the surface (Bruinink et al., 2015; Docter et al., 2015; Keene et al., 2012; Tenzer et al., 2013). Both processes are dynamic and depend on material properties as well as on the biological system and the exposure conditions. Models have been developed to describe the kinetics for the formation of a protein corona around nanoparticles and how this affects interspecies extrapolations of nanoparticle biodistribution (Dell'Orco et al., 2010; Sahneh et al., 2015; Vilanova et al., 2016). These models stress the importance of protein affinity, protein concentration, and time in the evolution of the corona. Uptake and transport of nanoparticles trough cell barriers occur *via* active and passive processes where some processes may be capacity limited (saturable), making the biokinetics nonlinear. Modeling cellular uptake can give valuable insight into such capacity limited processes. The outcome from these models suggests that receptor binding and recirculation of receptors are rate limiting processes (Lunov et al., 2011; Ohta et al., 2012). Despite progress, reported values on uptake capacity and uptake rate constant differs substantially between models describing *in vitro* experimental data. Models for endothelial translocation *in vivo* and the recognition and adaption of the mononuclear phagocyte system to nanoparticles are still largely lacking (Sarin, 2010). However, Li et al. (2016) performed PBPK modelling for inhaled fresh and aged cerium oxide nanoparticles and the model predicted the biodistribution well and identified phagocytizing cells in the pulmonary region as being responsible for capturing most of the nanoparticles not eliminated by feces (Figure 7). Lymphatic transport of nanoparticles has been demonstrated, but has rarely been measured in biodistribution studies or covered by PBPK models (Choi et al., 2010). This pathway has been stated to be important to be involved when NPs are translocated from the lung and gastrointestinal tract to the systemic circulation. The lymphatic transport has also been said to increase with dose, but the kinetic mechanism is unclear and requires further attention. Dissolution is another process that has been implemented in PBPK models for nanoparticles e.g.. for silver and zinc, but where empirical fitting rather than physiologically and physical principles have been used (Bachler et al., 2013; Chen et al., 2015; Utembe et al., 2015). A step forward would be to implement physically and physiologically relevant description of dissolution into the PBPK models. Several theoretical dissolution models for nanoparticles have been proposed suggesting that dissolution can follow different reaction kinetics. However, which reaction kinetics to use is still unclear. *In vitro* and *in vivo* studies have shown that the dissolution rate depends on the chemical and physical properties (size, surface area, etc) of the particles as well as on

the biological milieu (composition, ionic strength, pH, temperature, etc). In addition, dissolved substances can have different properties, toxicity, and biokinetics compared with nanoparticles. Consequently, reaction kinetics may differ between nanoparticle type, exposure condition and biological system.

Concluding remarks

Understanding biokinetics is important for risk assessment. Nanoparticles interact with biological systems, leading to changes in their physiochemical and biological properties and this may alter their biokinetics. With improved understanding of the mechanism of translocation of nanoparticles across biological barriers and the influence of dissolution, agglomeration/aggregation, and bio-corona formation on biokinetics, parameters used for modeling can be refined to better reflect physical and physiological principles. Model development is hampered by limited experimental data suitable for modelling. In particular, repeated exposure and long term exposure studies of well-characerized ENMs are lacking.

References

Bachler G, von Goetz N, Hungerbuhler K. (2013).A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. Int J Nanomed, 8, 3365-82.

Bachler G, Losert S, Umehara Y, von Goetz N, Rodriguez-Lorenzo L, Petri-Fink A, Rothen-Rutishauser B, Hungerbuehler K. (2015).Translocation of gold nanoparticles across the lung epithelial tissue barrier: Combining in vitro and in silico methods to substitute in vivo experiments. Particle and Fibre Toxicology, 12, 18.

Balashazy I, Hofmann W, Heistracher T. (2003).Local particle deposition patterns may play a key role in the development of lung cancer. J Appl Physiol (1985), 94, 1719-25.

Borm P, Cassee FR, Oberdorster G. (2015).Lung particle overload: old school -new insights? Particle and Fibre Toxicology, 12, 10.

Bouwmeester H, Poortman J, Peters RJ, Wijma E, Kramer E, Makama S, Puspitaninganindita K, Marvin HJ, Peijnenburg AA, Hendriksen PJ. (2011).Characterization of translocation of silver nanoparticles and effects on whole-genome gene expression using an in vitro intestinal epithelium coculture model. ACS Nano, 5, 4091-103.

Braakhuis HM, Kloet SK, Kezic S, Kuper F, Park MV, Bellmann S, van der Zande M, Le Gac S, Krystek P, Peters RJ, Rietjens IM, Bouwmeester H. (2015).Progress and future of in vitro models to study translocation of nanoparticles. Archives of toxicology.

Brain JD, Knudson DE, Sorokin SP, Davis MA. (1976).Pulmonary Distribution of Particles Given by Intratracheal Instillation or by Aerosol Inhalation. Environmental Research, 11, 13-33.

Brandenberger C, Muhlfeld C, Ali Z, Lenz AG, Schmid O, Parak WJ, Gehr P, Rothen-Rutishauser B. (2010).Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles. Small, 6, 1669-78.

Bruinink A, Wang J, Wick P. (2015).Effect of particle agglomeration in nanotoxicology. Archives of Toxicology, 89, 659-75.

Brun E, Barreau F, Veronesi G, Fayard B, Sorieul S, Chaneac C, Carapito C, Rabilloud T, Mabondzo A, Herlin-Boime N, Carriere M. (2014).Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia. Particle and Fibre Toxicology, 11.

Canton I, Battaglia G. (2012).Endocytosis at the nanoscale. Chemical Society Reviews, 41, 2718-39.

Cao Y, Li J, Liu F, Li XY, Jiang Q, Cheng SS, Gu YX. (2016).Consideration of interaction between nanoparticles and food components for the safety assessment of nanoparticles following oral exposure: A review. Environ Toxicol Phar, 46, 206-10.

Chen WY, Cheng YH, Hsieh NH, Wu BC, Chou WC, Ho CC, Chen JK, Liao CM, Lin P. (2015).Physiologically based pharmacokinetic modeling of zinc oxide nanoparticles and zinc nitrate in mice. Int J Nanomed, 10, 6277-92.

Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV. (2007).Renal clearance of quantum dots. Nat Biotechnol, 25, 1165-70.

Choi HS, Ashitate Y, Lee JH, Kim SH, Matsui A, Insin N, Bawendi MG, Semmler-Behnke M, Frangioni JV, Tsuda A. (2010).Rapid translocation of nanoparticles from the lung airspaces to the body. Nature biotechnology, 28, 1300-3.

Dell'Orco D, Lundqvist M, Oslakovic C, Cedervall T, Linse S. (2010).Modeling the time evolution of the nanoparticle-protein corona in a body fluid. PLoS One, 5, e10949.

DeLoid GM, Cohen JM, Pyrgiotakis G, Pirela SV, Pal A, Liu J, Srebric J, Demokritou P. (2015).Advanced computational modeling for in vitro nanomaterial dosimetry. Part Fibre Toxicol, 12, 32.

DeLorenzo AJD. (1970). The olfactory neuron and the blood-brain barrier. In Wolstenholme GEW, Knight J. Taste and Smell in Vertebrates. London, J&A Churchill, 151–76.

des Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. (2006).Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. Journal of Controlled Release, 116, 1-27.

Di Silvio D, Rigby N, Bajka B, Mackie A, Bombelli FB. (2016).Effect of protein corona magnetite nanoparticles derived from bread in vitro digestion on Caco-2 cells morphology and uptake. Int J Biochem Cell B, 75, 212-22.

Docter D, Westmeier D, Markiewicz M, Stolte S, Knauer SK, Stauber RH. (2015).The nanoparticle biomolecule corona: lessons learned - challenge accepted? Chemical Society Reviews, 44, 6094-121.

Dorier M, Brun E, Veronesi G, Barreau F, Pernet-Gallay K, Desvergne C, Rabilloud T, Carapito C, Herlin-Boime N, Carriere M. (2015).Impact of anatase and rutile titanium dioxide nanoparticles on uptake carriers and efflux pumps in Caco-2 gut epithelial cells. Nanoscale, 7, 7352-60.

Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J, Oberdorster G. (2006).Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. Environ Health Persp, 114, 1172-8.

Filon FL, Mauro M, Adami G, Bovenzi M, Crosera M. (2015).Nanoparticles skin absorption: New aspects for a safety profile evaluation. Regul Toxicol Pharm, 72, 310-22.
Filon FL, Crosera M, Adami G, Bovenzi M, Rossi F, Maina G. (2011).Human skin penetration of gold nanoparticles through intact and damaged skin. Nanotoxicology, 5, 493-501.
Filon FL, Bello D, Cherrie JW, Sleeuwenhoek A, Spaan S, Brouwer DH. (2016).Occupational dermal exposure to nanoparticles and nano-enabled products: Part I-Factors affecting skin absorption. Int J Hyg Environ Health, 219, 536-44.
Filon FL, Crosera M, Timeus E, Adami G, Bovenzi M, Ponti J, Maina G. (2013).Human skin penetration of cobalt nanoparticles through intact and damaged skin. Toxicology in Vitro, 27, 121-27.
Frohlich E, Salar-Behzadi S. (2014).Toxicological assessment of inhaled nanoparticles: role of in vivo, ex vivo, in vitro, and in silico studies. Int J Mol Sci, 15, 4795-822.
Frohlich E, Roblegg E. (2016).Oral uptake of nanoparticles: human relevance and the role of in vitro systems. Archives of Toxicology, 90, 2297-314.
George I, Vranic S, Boland S, Courtois A, Baeza-Squiban A. (2015).Development of an in vitro model of human bronchial epithelial barrier to study nanoparticle translocation. Toxicology in Vitro, 29, 51-58.
Geraets L, Oomen AG, Krystek P, Jacobsen NR, Wallin H, Laurentie M, Verharen HW, Brandon EF, de Jong WH. (2014).Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Particle and Fibre Toxicology, 11, 30.
Gustafson HH, Holt-Casper D, Grainger DW, Ghandehari H. (2015).Nanoparticle uptake: The phagocyte problem. Nano Today, 10, 487-510.
Gustafsson A, Bucht A, Österlund L. (2016). Interactions between inhaled nanomaterials and biomolecules in the lung – a study of the risks of systemic effectsed.
Hillyer JF, Albrecht RM. (2001).Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. J Pharm Sci, 90, 1927-36.
Hofmann W. (2011).Modelling inhaled particle deposition in the human lung-A review. J Aerosol Sci, 42, 693-724.
Iavicoli I, Fontana L, Nordberg G. (2016).The effects of nanoparticles on the renal system. Critical Reviews in Toxicology, 46, 490-560.
Iversen TG, Skotland T, Sandvig K. (2011).Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies. Nano Today, 6, 176-85.
Jani PU, Mccarthy DE, Florence AT. (1994).Titanium-Dioxide (Rutile) Particle Uptake from the Rat Gi Tract and Translocation to Systemic Organs after Oral-Administration. Int J Pharmaceut, 105, 157-68.
Johanson G, Ed., Modeling of disposition. In: Reference Module in Biomedical Sciences, Elsevier, 2014.
Johanson G, Rauma M. (2008).Basis for skin notation. Part 1. Dermal penetration data for substances on the Swedish OEL list. Arbete och Hälsa, 42, 1-235.
Johanson G, Carlander U. (2016). Uptake and biodistribution of nanoparticles – a review. KEMI - Swedish Chemicals Agency.

Keene AM, Peters D, Rouse R, Stewart S, Rosen ET, Tyner KM. (2012).Tissue and cellular distribution of gold nanoparticles varies based on aggregation/agglomeration status. Nanomedicine, 7, 199-209.
Keller J, Wohlleben W, Ma-Hock L, Strauss V, Groters S, Kuttler K, Wiench K, Herden C, Oberdorster G, van Ravenzwaay B, Landsiedel R. (2014).Time course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. Archives of toxicology, 88, 2033-59.
Kermanizadeh A, Balharry D, Wallin H, Loft S, Moller P. (2015).Nanomaterial translocation-the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs-a review. Critical reviews in toxicology, 1-36.
Lai SK, Wang YY, Hanes J. (2009).Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliver Rev, 61, 158-71.
Lee JA, Kim MK, Paek HJ, Kim YR, Kim MK, Lee JK, Jeong J, Choi SJ. (2014).Tissue distribution and excretion kinetics of orally administered silica nanoparticles in rats. Int J Nanomed, 9, 251-60.
Lesniak A, Fenaroli F, Monopoli MP, Aberg C, Dawson KA, Salvati A. (2012).Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells. ACS Nano, 6, 5845-57.
Lesniak A, Salvati A, Santos-Martinez MJ, Radomski MW, Dawson KA, Aberg C. (2013).Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. Journal of the American Chemical Society, 135, 1438-44.
Li D, Morishita M, Wagner JG, Fatouraie M, Wooldridge M, Eagle WE, Barres J, Carlander U, Emond C, Jolliet O. (2016).In vivo biodistribution and physiologically based pharmacokinetic modeling of inhaled fresh and aged cerium oxide nanoparticles in rats. Particle and Fibre Toxicology, 13, 45.
Li M, Zou P, Tyner K, Lee S. (2017).Physiologically Based Pharmacokinetic (PBPK) Modeling of Pharmaceutical Nanoparticles. AAPS J, 19, 26-42.
Lunov O, Zablotskii V, Syrovets T, Rocker C, Tron K, Nienhaus GU, Simmet T. (2011).Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages. Biomaterials, 32, 547-55.
Mahler GJ, Esch MB, Tako E, Southard TL, Archer SD, Glahn RP, Shuler ML. (2012).Oral exposure to polystyrene nanoparticles affects iron absorption. Nature Nanotechnology, 7, 264-U1500.
Mahmoudi M, Bertrand N, Zope H, Farokhzad OC. (2016).Emerging understanding of the protein corona at the nano-bio interfaces. Nano Today, 11, 817-32.
Meiring JJ, Borm PJ, Bagate K, Semmler M, Seitz J, Takenaka S, Kreyling WG. (2005).The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung. Part Fibre Toxicol, 2, 3.
Midander K, Pan J, Wallinder IO, Leygraf C. (2007).Metal release from stainless steel particles in vitro-influence of particle size. J Environ Monit, 9, 74-81.
Midander K, Julander A, Kettelarij J, Liden C. (2016).Testing in artificial sweat - Is less more? Comparison of metal release in two different artificial sweat solutions. Regul Toxicol Pharm, 81, 381-86.
Midander K, Pan JS, Wallinder IO, Heim K, Leygraf C. (2007).Nickel release from nickel particles in artificial sweat. Contact Dermatitis, 56, 325-30.
Misra SK, Dybowska A, Berhanu D, Luoma SN, Valsami-Jones E. (2012).The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. The Science of the total environment, 438, 225-32.

Morfeld P, Bruch J, Levy L, Ngiewih Y, Chaudhuri I, Muranko HJ, Myerson R, McCunney RJ. (2015).Translational toxicology in setting occupational exposure limits for dusts and hazard classification - a critical evaluation of a recent approach to translate dust overload findings from rats to humans. Part Fibre Toxicol, 12, 3.
Morrow PE. (1988).Possible mechanisms to explain dust overloading of the lungs. Fund Appl Toxicol, 10, 369-84.
Morrow PE, Haseman JK, Hobbs CH, Driscoll KE, Vu V, Oberdorster G. (1996).The maximum tolerated dose for inhalation bioassays: toxicity vs overload. Fund Appl Toxicol, 29, 155-67.
Ohta S, Inasawa S, Yamaguchi Y. (2012).Real time observation and kinetic modeling of the cellular uptake and removal of silicon quantum dots. Biomaterials, 33, 4639-45.
Owens DE, 3rd, Peppas NA. (2006).Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharmaceut, 307, 93-102.
Park EJ, Park YK, Park K. (2009).Acute Toxicity and Tissue Distribution of Cerium Oxide Nanoparticles by a Single Oral Administration in Rats Toxicological Research KSOT, 25, 79-84.
Pauluhn J. (2014).Derivation of occupational exposure levels (OELs) of Low-toxicity isometric biopersistent particles: how can the kinetic lung overload paradigm be used for improved inhalation toxicity study design and OEL-derivation? Particle and Fibre Toxicology, 11, 72.
Saba TM. (1970).Physiology and physiopathology of the reticuloendothelial system. Archives of Internal Medicine, 126, 1031-52.
Sahneh FD, Scoglio CM, Monteiro-Riviere NA, Riviere JE. (2015).Predicting the impact of biocorona formation kinetics on interspecies extrapolations of nanoparticle biodistribution modeling. Nanomedicine (Lond), 10, 25-33.
Sarin H. (2010).Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability. Journal of Angiogenesis Research, 2, 14.
Schleh C, Semmler-Behnke M, Lipka J, Wenk A, Hirn S, Schaffler M, Schmid G, Simon U, Kreyling WG. (2012).Size and surface charge of gold nanoparticles determine absorption across intestinal barriers and accumulation in secondary target organs after oral administration. Nanotoxicology, 6, 36-46.
Schulze C, Schaefer UF, Ruge CA, Wohlleben W, Lehr CM. (2011).Interaction of metal oxide nanoparticles with lung surfactant protein A. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV, 77, 376-83.
Singh SP, Rahman MF, Murty US, Mahboob M, Grover P. (2013).Comparative study of genotoxicity and tissue distribution of nano and micron sized iron oxide in rats after acute oral treatment. Toxicol Appl Pharm, 266, 56-66.
Sinnecker H, Krause T, Koelling S, Lautenschlager I, Frey A. (2014).The gut wall provides an effective barrier against nanoparticle uptake. Beilstein journal of nanotechnology, 5, 2092-101.
Sonavane G, Tomoda K, Sano A, Ohshima H, Terada H, Makino K. (2008).In vitro permeation of gold nanoparticles through rat skin and rat intestine: Effect of particle size. Colloid Surface B, 65, 1-10.
Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. (2015).TEER Measurement Techniques for In Vitro Barrier Model Systems. Jala-J Lab Autom, 20, 107-26.

Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. (2007).Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicological sciences : an official journal of the Society of Toxicology, 95, 300-12.

Tenzer S, Docter D, Kuharev J, Musyanovych A, Fetz V, Hecht R, Schlenk F, Fischer D, Kiouptsi K, Reinhardt C, Landfester K, Schild H, Maskos M, Knauer SK, Stauber RH. (2013).Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nature Nanotechnology, 8, 772-81.

Tinkle SS, Antonini JM, Rich BA, Roberts JR, Salmen R, DePree K, Adkins EJ. (2003).Skin as a route of exposure and sensitization in chronic beryllium disease. Environ Health Persp, 111, 1202-8.

Tran CL, Buchanan D, Cullen RT, Searl A, Jones AD, Donaldson K. (2000).Inhalation of poorly soluble particles. II. Influence Of particle surface area on inflammation and clearance. Inhalation toxicology, 12, 1113-26.

Utembe W, Potgieter K, Stefaniak AB, Gulumian M. (2015).Dissolution and biodurability: Important parameters needed for risk assessment of nanomaterials. Particle and Fibre Toxicology, 12, 11.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, Chai Z. (2007).Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett, 168, 176-85.

Wang YL, Yuan LL, Yao CJ, Ding L, Li CC, Fang J, Sui KK, Liu YF, Wu MH. (2014).A combined toxicity study of zinc oxide nanoparticles and vitamin C in food additives. Nanoscale, 6, 15333-42.

Vilanova O, Mittag JJ, Kelly PM, Milani S, Dawson KA, Radler JO, Franzese G. (2016).Understanding the Kinetics of Protein-Nanoparticle Corona Formation. ACS Nano, 10, 10842-50.

Win KY, Feng SS. (2005).Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials, 26, 2713-22.

Yang K, Gong H, Shi X, Wan J, Zhang Y, Liu Z. (2013).In vivo biodistribution and toxicology of functionalized nano-graphene oxide in mice after oral and intraperitoneal administration. Biomaterials, 34, 2787-95.

Yang W, Peters JI, Williams RO. (2008).Inhaled nanoparticles - A current review. Int J Pharmaceut, 356, 239-47.

4. Hazard Assessment

Nanomaterials may gain access to the body *via* different routes including through inhalation, ingestion, or dermal exposure. Once inside the body, nanomaterials may interact with cells and tissues, including organs distal from the portal of entry, potentially leading to adverse effects on multiple organs (Fadeel et al., 2017). In the following sections, the impact of three main classes of ENMs, i.e., carbon-based, metal, and metal oxide nanomaterials on key organ systems including the pulmonary system, cardiovascular system, skin, gastro-intestinal system, immune system, and central nervous system is presented. We will also discuss developmental/reproductive effects of ENMs, and carcinogenicity of ENMs.

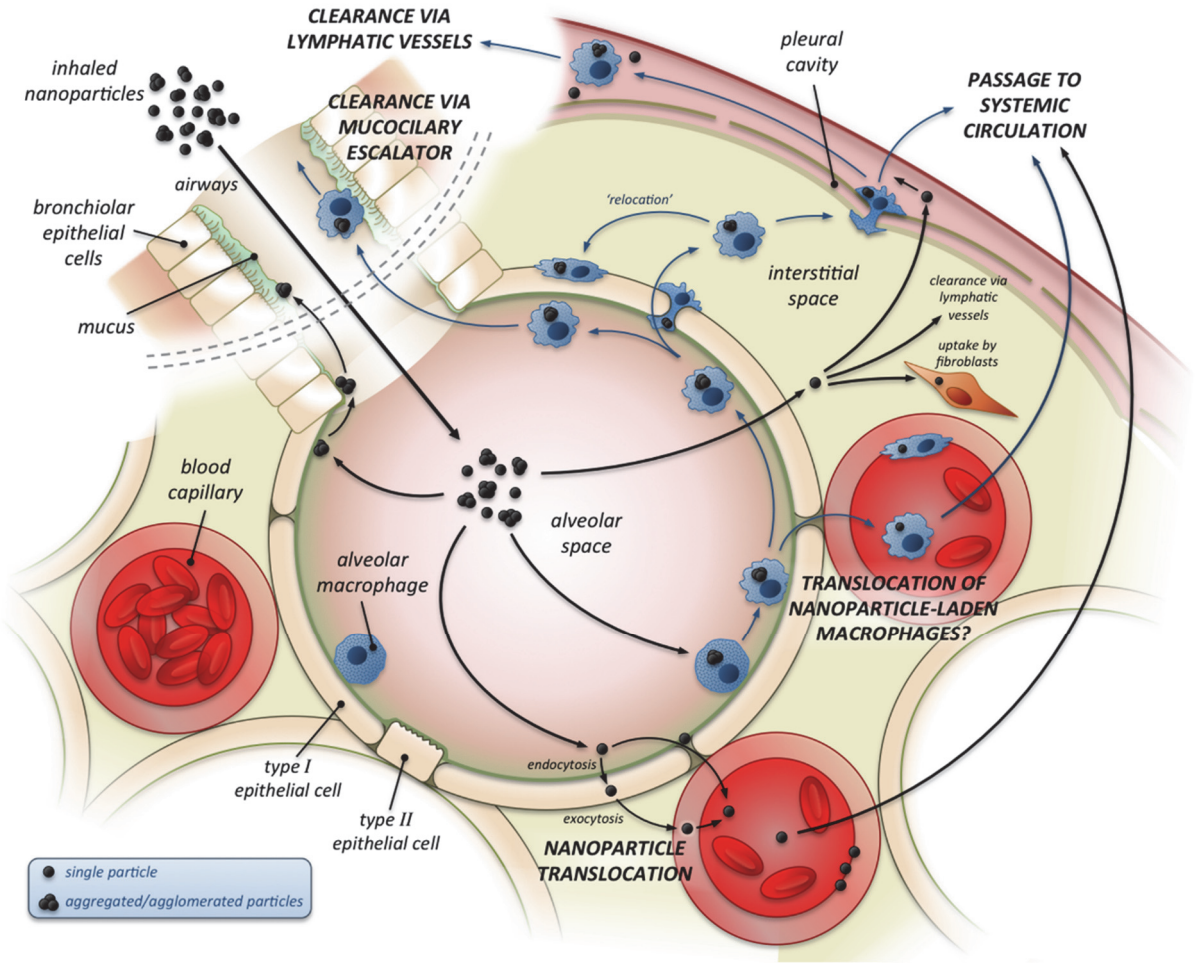


Figure 8. Pulmonary exposure to nanomaterials. Animal studies have shown that pathways exist for the transport of inhaled ENMs into the alveolar epithelium and interstitium and across the endothelial vascular barrier into the systemic circulation potentially allowing the materials to reach other organs in the body. From: Stone et al., 2017.

4.1. Pulmonary Toxicity of Nanomaterials

Upon deposition in the lungs, nanoparticles can potentially cause a range of pulmonary responses including inflammation, oxidative stress, fibrosis, genotoxicity, and cancer. Nanoparticles are taken up by macrophages in the lung with little inflammatory effects (Poland and Donaldson, 2017). However, if there is damage or stress to the macrophage, production of various cytokines and chemokines will follow leading to inflammation as a consequence. Moreover, animal studies have shown that pathways exist for the transport of inhaled ENMs into the alveolar epithelium and interstitium and across the endothelial vascular membrane into the systemic circulation thereby allowing the nanomaterials to reach other organs in the body (Stone et al., 2017). For low-toxicity, low-solubility materials, such as TiO_2 nanoparticles, the surface area appears to be the metric that mainly dictates inflammatory response (Duffin et al., 2007). For more reactive nanoparticles, the extent of surface reactivity and the toxicity of possible released ions are more likely to govern the effect (Cho et al., 2012).

Persistent inflammation is believed to be a risk factor for the development of fibrosis, characterized by an accumulation of collagen and fibroblast proliferation. Work related to CNT-induced fibrosis suggested inflammatory-driven effects as well as direct effects of CNTs on the fibroblasts (Labib et al., 2016; Vietti et al., 2016). Metal oxides such as CeO_2 have also been shown to induce sustained pulmonary inflammatory and modification of the balance of mediators involved in tissue repair processes leading to pulmonary fibrosis in the exposed lungs (Ma et al., 2012).

For regulatory purposes, the effects of a substance should be assessed following subacute (28-day) or subchronic (90-day) inhalation tests according to OECD guidelines, TG 412 and 413. However, as suggested by the OECD Working Party on Manufactured Nanomaterials (WPMN), a short-term inhalation study (STIS) in rats may be suitable to reduce and refine repeated-dose sub-acute inhalation toxicity testing. In such a study rats are exposed for 5 days, 6 h/day, and there should be a post-exposure observation (or recovery) period of up to 13 weeks. Lung burden assessments and various bronchoalveolar lavage parameters are used. The STIS can provide information on early events of nanoparticle-induced pathogenesis, such as acute lung damage and inflammation, as well as on the reversibility, persistence or progression of effects. Furthermore, lung burden and potential for extra-pulmonary translocation may be

investigated in the STIS (Landsiedel et al., 2014). However, it is more difficult to predict subchronic toxicity endpoints, such as fibrosis and cancer. Due to the laborious nature of inhalation studies, other techniques are often used, such as intratracheal instillation and pharyngeal aspiration.

Metal and metal oxide nanoparticles

Different metal and metal oxide nanoparticles have been tested in a range of *in vitro* studies as well as *in vivo* (Karlsson et al., 2014). Here, some examples of studies will be discussed with a focus on studies comparing the potency of various metal and metal oxide nanoparticles. Donaldson and co-workers investigated the acute inflammation in rat lungs following instillation of 15 different metal/metal oxide nanoparticles (Cho *et al.*, 2012). The same surface area dose of the nanoparticles (150 cm^2) was used and effects were studied 24 h after exposure. Many of the tested nanoparticles were highly inflammatory at the dose tested. The results showed also that the inflammatory potential appeared to be significantly correlated with one of two physicochemical parameters: zeta potential under acid conditions for low-solubility nanoparticles and degree of dissolution for high-solubility nanoparticles. Thus, in the case of high-solubility nanoparticles, toxicity depends on the ions that are released in the acidic lysosomes. If the ions are toxic (as is the case for Cu and Zn ions) the lysosomes will be destabilized and this will lead to an inflammatory response (see chapter on immunotoxicity). High acute toxicity of CuO and ZnO nanoparticles has also been observed in *in vitro* studies using cultured lung cells (Karlsson et al., 2008; Lanone et al., 2009). For low-solubility particles, on the other hand, there was a correlation between their zeta potential at acidic condition and their potency for causing lung inflammation (Cho et al., 2012). It should be noted that the latter study focused on acute effects after a single dose of nanoparticles. Indeed, the biopersistence and degree of reversibility are important factors that need to be taken into account in risk assessment of ENMs.

Landsiedel et al. (2017) compared the potencies of various metal and metal oxide nanoparticles based on short-term inhalation study (STIS) results. The NOAEC (No Observed Adverse Effect Concentration) observed in the studies was highlighted as well as the degree of reversibility. Interestingly, low NOAECs (0.5 mg/m^3 or lower based upon respiratory tract effects) and thus high toxicity, were found for ZnO (NM-111), CeO_2 (NM-211 and NM-212), as well as quartz dust (DQ12 micron-sized included as comparison). CuO nanoparticles also displayed high

toxicity (NOAEL 0.6 mg/m^3). The effects observed for the CeO_2 nanoparticles (and quartz) were progressive whereas those for ZnO NM-11 and CuO were fully reversible within the 3-week post-exposure period. Nanoparticles with high biopersistence in the lung (i.e., pulmonary half-life >40 days) were TiO_2 (NM-105), SiO_2 acrylate, the two CeO_2 materials (NM-211 and NM-212), as well as the quartz dust. For rather non-soluble metal oxide nanoparticles, the semiconducting properties could be of high importance and thus, the oxidative stress potential and hence toxicity may be predicted by comparing their conduction and valence band energy levels with redox potentials of biological reactions occurring in the cells (Burello and Worth, 2011). If these two energy levels are comparable, then electrons can be transferred and the oxide acts like a catalyst or as an electron donor or acceptor. Nel and co-workers tested this 'band-gap theory' using 24 different metal oxide nanoparticles both *in vitro* and *in vivo* (Zhang et al., 2012). They found that the overlap of conduction band energy (Ec) levels with the cellular redox potential (−4.12 to −4.84 eV) was correlated to the ability of Co_3O_4, Cr_2O_3, Ni_2O_3, Mn_2O_3, and CoO nanoparticles to induce oxygen radicals, oxidative stress, and inflammation. The authors concluded that: (1) the toxicity of metal oxide nanoparticles can be traced to the catalytic properties of the intact particles as well as their ability to release toxic metal ions; (2) particle catalytic effects and dissolved metal ions contribute to ROS generation and oxidative stress injury; and (3) particulate-induced oxidative stress in response to nanoparticles, as well as ambient ultrafine particles, is closely related to the generation of pulmonary inflammation (Zhang et al., 2012).

The most well studied metallic nanoparticle type is nano-Ag. Weldon et al. (2016) recently compiled relevant inhalation studies in rats in order to be able to propose OEL values for silver. They concluded that a 90-day inhalation study was most important for risk assessment (Sung et a*l., 200*9) and this study showed various lung effects including inflammation at the concentration 515 µg/m^3. However, Weldon et al. argued that the liver appeared to be the most sensitive target organ, and bile duct hyperplasia was regarded as a critical effect. Calculations led to a suggestion of an occupational exposure limit of 0.19 µg/m³ (Weldon et al., 2016).

Carbon-based materials

The pulmonary toxicity of carbon-based materials such as carbon black (CB) has been investigated in workers exposed occupationally as well as in a number of animal and *in vitro* studies. Carcinogenic effects of CB will

be discussed in the chapter focused on genotoxicity and cancer. Non-cancer respiratory effects in workers exposed to CB include cough, sputum production, bronchitis, chest radiographic opacities (e.g., pneumoconiosis) and decrements in lung function (IARC, 2010). The critical effect level for chronic pulmonary non-cancer effects by inhalation (LOEC) has been determined to be 0.57 mg/m^3, based on increased respiratory symptoms and decreased lung function measurements in individuals (male) exposed to CB in an occupational setting (ECHC, 2013). A number of toxic effects of CB have also been reported in experimental studies. These effects include inflammation, lung epithelial cell injury, DNA damage and oxidative stress (Bourdon et al., 2012; IARC, 2010). Concerning CNTs and carbon nanofibers (CNFs), NIOSH published a comprehensive bulletin in 2013 (NIOSH, 2013). In this report, NIOSH systematically reviewed 54 laboratory animal studies, and showed that many of these clearly indicated that CNT/CNF could cause adverse pulmonary effects including inflammation (44/54 studies), granulomas (27/54 studies), and pulmonary fibrosis (25/54 studies). Moreover, the report also concluded that in animal studies in which CNTs were compared with other known fibrogenic materials (including asbestos and ultrafine carbon black), the CNTs were of similar or greater potency. Based on the available data, NIOSH calculated a recommended exposure limit (REL) of 1 µg/m^3 of respirable elemental carbon (8 hr time-weighted average). When considering fibers the most well described mechanistic paradigm is the ‘fibre pathogenicity paradigm’. According to this paradigm, pathogenic fibers are characterized as being: (1) thin enough to allow deposition deep in the lungs; (2) long enough to cause “frustrated phagocytosis” and retention in the pleural space (i.e., not cleared to the lymph nodes); and (3) biopersistant leading to long retention in the lungs (Donaldson et al., 2013). One critical question is therefore whether or not CNTs conform to this paradigm. Clearly, CNTs can be manufactured as tight tangles, which more resemble particles, or as high aspect ratio fibres. The effect of the ‘particle-like’ CNTs is more likely to be similar to carbon-based particles (respiratory effects possibly including fibrosis and lung cancer) whereas fibre-like CNTs could have pulmonary effects but may also affect the pleura (fibrosis and mesothelioma). Regarding biopersistance, pristine CNTs are generally considered to be durable materials, but there is evidence that highly oxidized SWCNTs can be further broken down by peroxidase enzymes expressed in neutrophils (Kagan et al., 2010) or *via* an oxidative process that depends on NADPH oxidase-driven production of superoxide anion radicals and NO·radicals produced by the nitric oxide synthase in macrophages (Kagan et al., 2014). SWCNTs coated or functionalized with PEG chains have also been shown to undergo biodegradation by myeloperoxidase (MPO) in an acellular system, and

were degraded by *ex vivo* activated primary human neutrophils (Bhattacharya et al., 2014). Apart from the 'fibre-like' properties of CNTs, other physicochemical properties which may determine the biological reactivity of CNTs include: (1) wall number (e.g., SWCNT or MWCNT, which results in different diameters and flexibility); (2) presence of various metals (often used as catalysts during preparation); (3) extent of surface defects; (4) shape (straight, curled or entangled); (5) degree of functionalization (i.e., surface modifications such as oxidation or PEGylation); and (6) formation of different bio-coronas (Kuempel et al., 2017). Based on recent theoretical modeling studies, an additional factor, mechanical bending stiffness, has been suggested as being predictive of the potential carcinogenicity of carbon-based nanomaterials including CNTs (Zhu et al., 2016). According to this paradigm, stiff nanotubes beyond a critical length are compressed by lysosomal membranes causing persistent contact with the inner membrane, leading to lipid extraction, lysosomal permeabilization, release of cathepsin B (a lysosomal protease) into the cytoplasm, and cell death. In a recent study, ten different MWCNTs were investigated with the aim to identify potential physicochemical drivers of MWCNT-induced inflammation and genotoxicity following intratracheal instillation into rats (Poulsen et al., 2016). The results showed that all materials induced dose-dependent inflammation (as evidenced by an increased number of neutrophils in bronchoalveolar lavage fluid), and surface area (BET) appeared to be a positive predictor since the thin CNTs with large BET were found to be the most active. CNTs with larger diameter were associated with genotoxicity. In another recent study, Scala et al. (2018) performed genome-wide profiling of DNA methylation, mRNA and microRNA expression in three cell lines representative exposed to ten carbon-based nanomaterials. Using advanced data integration and modelling techniques in order to build regulatory and functional maps of the effects, the authors concluded that the molecular alterations were highly dependent on the geometrical properties of the nanoparticles as well as on the cell type. However, the number of studies investigating potential effects in workers exposed to CNTs is limited. A recent study including a small number of exposed workers (n=10) showed significantly elevated pro-fibrotic inflammatory mediators, including IL-1b, IL-4, IL-10, and TNFα, in sputum and serum samples when compared to unexposed controls (Fatkhutdinova et al., 2016).

"mechanical bending stiffness has been suggested as being predictive of the carcinogenicity of carbon-based nanomaterials"

The graphene-based family of materials including graphene and graphene oxide (GO) has also received increasing attention in recent years. Ema et al. (2017) recently compiled a review of animal studies of these materials. Four inhalation studies in rats were found and 2/3 studies on graphene reported inflammation in the lungs at exposure concentrations approx. >3 mg/m^3 (for 6 h/day for 5 days). The negative study tested up to 1.88 mg/m^3 but in this case for 4 weeks (6 h/day, 5 days/week). The study on GO reported inflammation following a single 6 h exposure starting from 3.76 mg/m^3. In addition to these studies, eight studies using mice and one study on rats exposed by intratracheal instillation or pharyngeal aspiration were summarized. More recently, researchers in the European Commission-funded Graphene Flagship have provided a state-of-the-art review of safety assessment of graphene-based materials and highlighted the importance of careful characterization of the test material (Fadeel et al., 2018). The lateral dimensions of graphene oxide sheets seem to play an important role for the biological reactivity of this material (Mukherjee et al., 2018).

References

Bhattacharya, K., Sacchetti, C., El-Sayed, R., Fornara, A., Kotchey, G. P., Gaugler, J. A., Star, A., Bottini, M., and Fadeel, B. (2014). Enzymatic 'stripping' and degradation of PEGylated carbon nanotubes. Nanoscale 6(24), 14686-90.

Bourdon, J. A., Saber, A. T., Jacobsen, N. R., Jensen, K. A., Madsen, A. M., Lamson, J. S., Wallin, H., Moller, P., Loft, S., Yauk, C. L., et al. (2012). Carbon black nanoparticle instillation induces sustained inflammation and genotoxicity in mouse lung and liver. Particle and fibre toxicology 9, 5.

Burello, E., and Worth, A. P. (2011). A theoretical framework for predicting the oxidative stress potential of oxide nanoparticles. Nanotoxicology 5(2), 228-35.

Cho, W. S., Duffin, R., Thielbeer, F., Bradley, M., Megson, I. L., Macnee, W., Poland, C. A., Tran, C. L., and Donaldson, K. (2012). Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal oxide nanoparticles. Toxicological sciences : an official journal of the Society of Toxicology 126(2), 469-77.

Donaldson, K., Poland, C. A., Murphy, F. A., MacFarlane, M., Chernova, T., and Schinwald, A. (2013). Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences. Advanced drug delivery reviews 65(15), 2078-86.

Duffin, R., Tran, L., Brown, D., Stone, V., and Donaldson, K. (2007). Proinflammogenic effects of low-toxicity and metal nanoparticles in vivo and in vitro: highlighting the role of particle surface area and surface reactivity. Inhalation toxicology 19(10), 849-56.

ECHC (2013). Screening Assessment for the Challenge: Carbon Black. Chemical Abstracts Service Registry Number 1333-86-4.

Ema, M., Gamo, M., and Honda, K. (2017). A review of toxicity studies on graphene-based nanomaterials in laboratory animals. Regulatory toxicology and pharmacology : RTP 85, 7-24.

Fadeel B, Pietroiusti A, Shvedova A. (eds). *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health*. Elsevier-Academic Press. Second Edition. (2017).

Fadeel B, et al. Safety assessment of graphene-based materials: focus on human health and the environment. ACS-Nano. 2018 [in press].

Fatkhutdinova, L. M., Khaliullin, T. O., Vasil'yeva, O. L., Zalyalov, R. R., Mustafin, I. G., Kisin, E. R., Birch, M. E., Yanamala, N., and Shvedova, A. A. (2016). Fibrosis biomarkers in workers exposed to MWCNTs. Toxicology and applied pharmacology 299, 125-31.

IARC (2010). Carbon Black, Titanium Dioxide, and Talc. IARC MONOGRAPHS VOLUME 93.

Kagan, V. E., Kapralov, A. A., St Croix, C. M., Watkins, S. C., Kisin, E. R., Kotchey, G. P., Balasubramanian, K., Vlasova, II, Yu, J., Kim, K., et al. (2014). Lung macrophages "digest" carbon nanotubes using a superoxide/peroxynitrite oxidative pathway. ACS nano 8(6), 5610-21.

Kagan, V. E., Konduru, N. V., Feng, W., Allen, B. L., Conroy, J., Volkov, Y., Vlasova, II, Belikova, N. A., Yanamala, N., Kapralov, A., et al. (2010). Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nature nanotechnology 5(5), 354-9.

Karlsson, H. L., Cronholm, P., Gustafsson, J., and Moller, L. (2008). Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chemical research in toxicology 21(9), 1726-32.

Karlsson, H. L., Toprak, M., and Fadeel, B. (2014). Toxicity of metal and metal oxide nanoparticles. In: Handbook on Toxicity of Metals. Fourth Edition. 75-112.

Kuempel, E. D., Jaurand, M. C., Moller, P., Morimoto, Y., Kobayashi, N., Pinkerton, K. E., Sargent, L. M., Vermeulen, R. C., Fubini, B., and Kane, A. B. (2017). Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans. Critical reviews in toxicology 47(1), 1-58.

Labib, S., Williams, A., Yauk, C. L., Nikota, J. K., Wallin, H., Vogel, U., and Halappanavar, S. (2016). Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Particle and fibre toxicology 13, 15.

Landsiedel, R., Ma-Hock, L., Hofmann, T., Wiemann, M., Strauss, V., Treumann, S., Wohlleben, W., Groters, S., Wiench, K., and van Ravenzwaay, B. (2014). Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Particle and fibre toxicology 11, 16.

Landsiedel, R., Sauer, U.G., de Jong, W. (2017). Risk Assessment and Risk Management. In: *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health*. Fadeel B, Pietroiusti A, Shvedova A. (eds). Elsevier-Academic Press. Second Edition. (2017).

Lanone, S., Rogerieux, F., Geys, J., Dupont, A., Maillot-Marechal, E., Boczkowski, J., Lacroix, G., and Hoet, P. (2009). Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Particle and fibre toxicology 6, 14.

Ma, J. Y., Mercer, R. R., Barger, M., Schwegler-Berry, D., Scabilloni, J., Ma, J. K., and Castranova, V. (2012). Induction of pulmonary fibrosis by cerium oxide nanoparticles. Toxicology and applied pharmacology 262(3), 255-64.

Mukherjee SP, Lazzaretto B, Hultenby K, Newman L, Rodrigues AF, Lozano N, Kostarelos K, Malmberg P, Fadeel B. Graphene oxide elicits membrane lipid changes and neutrophil extracellular trap formation. Chem. 2018;4(2):334-58.

NIOSH (2013). Occupational Exposure to Carbon Nanotubes and Nanofibers. CURRENT INTELLIGENCE BULLETIN 65, Publication No. 2013–145.

Poland, C. A., and Donaldson, K. (2017). Respiratory System, Part One: Basic Mechanisms. Adverse Effects of Engineered Nanomaterials. 225-42.

Poulsen, S. S., Jackson, P., Kling, K., Knudsen, K. B., Skaug, V., Kyjovska, Z. O., Thomsen, B. L., Clausen, P. A., Atluri, R., Berthing, T., et al. (2016). Multi-walled carbon nanotube physicochemical properties predict pulmonary inflammation and genotoxicity. Nanotoxicology 10(9), 1263-75.

Scala G, Kinaret P, Marwah V, Sund J, Fortino V, Greco D. Multi-omics analysis of ten carbon nanomaterials effects highlights cell type specific patterns of molecular regulation and adaptation NanoImpact. 2018;11:99-108.

Stone V, Miller MR, Clift MJD, Elder A, Mills NL, Møller P, Schins RPF, Vogel U, Kreyling WG, Alstrup Jensen K, Kuhlbusch TAJ, Schwarze PE, Hoet P, Pietroiusti A, De Vizcaya-Ruiz A, Baeza-Squiban A, Teixeira JP, Tran CL, Cassee FR. Nanomaterials *versus* ambient ultrafine particles: an opportunity to exchange toxicology knowledge. Environ Health Perspect. 2017;125(10):106002.

Sung, J. H., Ji, J. H., Park, J. D., Yoon, J. U., Kim, D. S., Jeon, K. S., Song, M. Y., Jeong, J., Han, B. S., Han, J. H., et al. (2009). Subchronic inhalation toxicity of silver nanoparticles. Toxicological sciences : an official journal of the Society of Toxicology 108(2), 452-61.

Weldon, B. A., E, M. F., Oberdorster, G., Workman, T., Griffith, W. C., Kneuer, C., and Yu, I. J. (2016). Occupational exposure limit for silver nanoparticles: considerations on the derivation of a general health-based value. Nanotoxicology 10(7), 945-56.

Vietti, G., Lison, D., and van den Brule, S. (2016). Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). Particle and fibre toxicology 13, 11.

Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., Pokhrel, S., Lin, S., Wang, X., Liao, Y. P., et al. (2012). Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS nano 6(5), 4349-68.

Zhu W, von dem Bussche A, Yi X, Qiu Y, Wang Z, Weston P, Hurt RH, Kane AB, Gao H. Nanomechanical mechanism for lipid bilayer damage induced by carbon nanotubes confined in intracellular vesicles. Proc Natl Acad Sci U S A. 2016;113(44):12374-12379.

4.2. Cardiovascular Toxicity of Nanomaterials

The relationship between exposure to increased levels of ambient particulate matter with higher mortality and morbidity rates due to cardiovascular impairments are already well-established (Fadeel et al., 2007; Mills et al., 2007). Some understanding of ENM effects can be derived from previous studies of ultrafine particles, which have been shown to produce acute/chronic pulmonary inflammation, oxidative stress response, systemic inflammation due to prolonged pulmonary inflammation, and procoagulant changes in the lung and in systemic site and may finally lead to the observed cardiovascular effect (Mills et al., 2007). Ultrafine particles are defined as air-borne particles smaller than 100 nm and are the products of combustion and vaporization processes. Furthermore, ambient ultrafine particles are heterogeneous in size, aggregation state and in chemical composition, all of which can contribute to ROS generation and toxicity (Simeonova et al., 2010). Newly emerging ENMs, though similar in size to ultrafine particles, may differ greatly in their chemical composition. Nevertheless, based on studies of ultrafine particles, one may postulate that localized pulmonary inflammatory and oxidative stress responses could lead to systemic effects through alterations of the blood coagulation cascade, thereby causing atherogenic reactions and cardiac impairments. This paradigm should be investigated under experimental conditions in order to prevent potential ENM toxicity towards the cardiovascular system resulting from occupational and environmental exposure to ENMs. Direct translocation from the lungs to systemic circulation also needs to be taken into consideration (Mills et al., 2006).

Carbon nanotubes

SWCNTs may cause pulmonary inflammation and fibrosis in mice (Shvedova et al., 2005). Furthermore, pulmonary exposure to SWCNTs may also lead to cardiovascular effects (Li et al., 2007). Hence a single intrapharyngeal instillation of SWCNTs induced activation of heme oxygenase-1 (HO-1), a known biomarker of oxidative stress (Prawan et al. 2005), in lung, aorta, and heart tissue in HO-1 reporter transgenic mice (Li et al., 2007). Furthermore, mice exposed to SWCNT (10 and 40 µg/mouse), developed aortic mtDNA damage and this accompanied by changes in aortic mitochondrial glutathione and protein carbonyl levels. The authors also showed that repeated exposure to SWCNTs (20 µg/mouse once every other week for 8 weeks) stimulated the progression of atherosclerosis in Apolipoprotein E-deficient ($ApoE^{-/-}$) transgenic mice. Although SWCNT exposure did not modify the lipid profiles of these mice, it resulted in accelerated plaque formation in $ApoE^{-/-}$ mice fed an

atherogenic diet (Li et al., 2007). Thus, a plausible molecular mechanism might be that pulmonary toxicity following prolonged deposition of SWCNTs in the lungs is associated with cardiovascular effects through an increased oxidative stress, which can result in altered vessel homeostasis. This study is further supported by the study of Erdely et al. (2009), showing clear signs of oxidative stress and inflammation due to upregulation of HO-1 expression in aorta following pharyngeal aspiration of SWCNTs or MWCNTs. In a follow-up study, the authors demonstrated that exposure to MWCNTs by pharyngeal aspiration resulted in systemic inflammation as observed by increased the serum and liver levels of acute phase proteins (Erdely et al., 2011). Poulsen et al. 2017 investigated the physicochemical determinants of MWCNT-induced systemic acute phase responses by analyzing the effects of pulmonary exposure to 14 different MWCNTs. The results revealed differential pulmonary and hepatic acute phase responses after MWCNT exposure. Christophersen et al. (2016) investigated the cardiovascular and pulmonary effects following repeated oral or pulmonary exposures to MWCNTs in $ApoE^{-/-}$ mice fed a Western-type diet. The authors found that repeated pulmonary exposure to MWCNTs resulted in cardiovascular effects with remodelling of the aorta wall. However, they did not find accelerated plaque progression in the aorta. Ge et al. (2012) reported that intratracheal instillation exposure of spontaneously hypertensive rats (SHRs) to SWCNTs (0.6 mg/rat) resulted in acute pulmonary inflammatory response and cardiovascular impairments as evidenced by endothelial dysfunction, which was associated with increased serum concentration of vasoconstriction factors endothelin 1 and angiotensin I converting enzyme as well as thickening of arterial vessels. The authors suggested that individuals with preexisting complications (e.g., hypertension, chronic inflammation) may be more susceptible to SWCNT exposure. Updahyay et al. (2008, 2014) reported similar responses following inhalation exposure of adult and aged SHRs to ultrafine carbon particles. In these studies, the authors clearly showed that aged SHRs are susceptible for the carbon nanoparticle-mediated onset of cardiovascular system impairments compared to adult SHRs. Interestingly, Increased blood pressure and heart rate were detected in SHRs exposed to the ultrafine carbon particles while pulmonary and blood systemic inflammatory markers were not affected (Updahyay et al., 2008). Thus, nanoparticles may cause cardiovascular and pulmonary impairment in the absence of detectable pulmonary inflammation, and this may be of particular relevance in individuals with preexisting cardiovascular diseases.

The question is whether the translocation of ENMs to extrapulmonary sites could also play a role for the cardiovascular effects. Several studies have

shown the translocation of ENMs to extrapulmonary sites (circulation, liver, placenta etc) (Kreyling et al., 2009, Mills et al., 2006, Möller et al., 2008). Nemmar et al. (2007) reported that mild CNT-induced lung inflammation translates *via* transient activation of platelets into P-selectin-mediated systemic inflammation in exposed rats, in turn eliciting inflammation-induced procoagulant activity and an associated prothrombotic risk. Furthermore, *in vitro* studies have provided evidence of potential effects on human aortic and microvascular endothelial cells exposed to SWCNTs or MWCNTs, with increased the release of pro-inflammatory cytokines and cell adhesion molecules (Pacurari et al., 2012; Walker et al., 2009, Vidanapathiran et al., 2012). Moreover, these studies showed that the exposure to CNTs resulted in disruption of the cytoskeleton and cell–cell interactions, which may is associated with increased permeability, possibly resulting in elevated translocation of particles across cell barriers.

Metal oxide nanoparticles

Detailed in vitro testing of different metal oxide nanoparticles (zinc oxide, iron oxide, magnesium oxide (MgO), aluminum oxide (Al_2O_3), and copper(II) oxide (CuO) using human cardiac microvascular endothelial cells revealed that cytotoxicity, permeability, and the expression of inflammatory markers depended on particle composition, concentration, and exposure time (Sun et al., 2011). ZnO, CuO, and MgO NPs resulted in more permeability and inflammation responses than other metal oxides.

Titanium dioxide particles (TiO_2) have been considered as nontoxic mineral particles and are regularly used in several commercial products like toothpastes, sunscreens, cosmetics, food and also in drugs. However, studies suggest that TiO_2 nanoparticles may elicit toxicity and might responsible for different cytotoxic outcomes (Chen et al., 2009; Shi et al., 2013). Repeated inhalation exposure of $ApoE^{-/-}$ mice to TiO_2 nanoparticles elicited moderate progression of plaque formation in the aorta, without showing any pulmonary inflammation and vasodilatory dysfunction (Mikkelsen et al., 2011). However, a sub chronic intra-tracheal instillation exposure of $ApoE^{-/-}$ mice to TiO_2 (10, 50 and 100 µg) showed Our study showed that tracheal instillation of nano-TiO_2 particles induced considerable systemic inflammation, endothelial dysfunction and lipid metabolism dysfunction, contributing to the progression of atherosclerosis (Chen et al., 2013). Chronic oral exposure of Sprague-Dawley rats to TiO_2 (0, 2, 10, 50 mg/kg for 30 and 90 days) showed that even at a low dose, TiO_2 nanoparticles can induce adverse cardiovascular effects in association with significant systemic inflammatory responses as indicated by secretion of TNF-α and IL-6 (Chen et al., 2015). Similarly, chronic exposure (6 months) of CD-1 mice to TiO_2 nanoparticles resulted in

cardiac lesions coupling with pulmonary inflammation and systemic inflammation characterized by induced level of C-reactive protein, and other pro-inflammatory factors both in pulmonary tissue and in serum by Hong et al. (2015). Rueda-Romero et al. (2016) showed activation of human monocytes by TiO_2 nanoparticles at very low concentrations as a result of induced oxidative stress. Chen et al. (2008) reported pulmonary and cardiovascular effects in young, adult and aged rats following sub chronic exposure to aerosolized SiO_2 nanoparticles. In this study, inhaled SiO_2 nanoparticles significantly increased pulmonary inflammation, myocardial ischemic damage, and atrio-ventricular blockage. The authors concluded that the observed pulmonary and cardiovascular effect of SiO_2 were more significant in old individuals than in young and adult rats. Detailed *in vitro* studies of SiO_2 nanoparticles showed that exposure of endothelial cells resulted in the perturbation of the NO/NOS system, and induction of inflammatory responses, with potential endothelial dysfunction *via* the PI3K/Akt/mTOR pathway (Duan et al., 2014). El-Hussainy et al. (2016) recently reported that chronic exposure of rats (daily intraperitoneal injection for 14 days) to Al_2O_3 nanoparticles caused myocardial dysfunction, inflammation, and oxidative stress, with downregulation of connexin 43 (Cx43) expression in the heart. Cx43 is the predominant protein forming gap junctions in rat ventricular myocardium, and its expression is critical for maintaining normal cardiac electrical conduction.

“Based on ambient ultrafine particle research, it is predicted that nanoparticles may display deeper pulmonary deposition and a tendency for extra-pulmonary translocation compared to larger particles.”

Metal nanoparticles

Kang et al. (2011) reported a clear association between long-term inhalation exposure of a susceptible mouse model ($ApoE^{-/-}$) to metal nanoparticles and the onset of cardiovascular disease. Exposure of human vascular endothelial cells (HUVEC) to SiO_2 nanoparticles induces VEGFR2-mediated autophagic activity, which may play a critical role in maintaining endothelium and vascular homeostasis. Moreover, SiO_2 expsoure may trigger alteration of angiogenesis in HUVECs (Duan et al., 2014).

Concluding remarks

Taken together, the abovementioned findings are of sufficient significance to warrant further studies to evaluate test approaches for prediction of

potential systemic toxicity following respiratory exposure to various ENMs. Based on ambient ultrafine particle research, it is predicted that ENMs may display deeper pulmonary deposition, higher biological activity/potency, and a greater tendency for extra-pulmonary translocation compared to larger particles of the same composition. In this regard, pulmonary exposure to ENMs, through direct or indirect mechanisms, may lead to unexpected distal responses involving the immune system and the cardiovascular system, or other organ systems. The systemic effects may induce or modify the progression of existing diseases such as cardiovascular disease. Further studies to develop biomarkers of exposure to particles in order to predict potential extra-pulmonary toxicity, including cardiovascular disease, are seen as an important research priority.

References

Ballinger SW. 2002; Mitochondrial integrity and function in atherogenesis. Circulation. 106(5):544–549.

Ballinger SW. 2005. Mitochondrial dysfunction in cardiovascular disease. Free Radical Biol Med. 38(10):1278–1295.

Ballinger SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, et al. 2002. Mitochondrial integrity and function in atherogenesis. Circulation. 106(5):544–549.

Chen J et al. 2009; In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitioneal injection. Journal of applied toxicology, 29.4. 330-337.

Chen T et al. 2013; Cardiovascular effects of pulmonary exposure to titanium dioxide nanoparticles in ApoEknockout mice. J Nanosci Nanotechnol. 2013;13(5):3214-22.

Chen Z et al. 2008; Age-related differences in pulmonary and cardiovascular responses to SiO2. Environ Sci Technol 42 (23), 8985-8992.

Chen Z et al. 2015; Effect of titanium dioxide nanoparticles on the cardiovascular system after oral administration. Toxicol Lett 239 (2), 123-130. 2015.

Christophersen DV, Jacobsen NR, Andersen MH, Connell SP, Barfod KK, Thomsen MB, Miller MR, Duffin R, Lykkesfeldt J, Vogel U, Wallin H, Loft S, Roursgaard M, Møller P. Cardiovascular health effects of oral and pulmonary exposure to multi-walled carbon nanotubes in ApoE-deficient mice. Toxicology. 2016;371:29-40.

Duan J et al. 2014; Silica nanoparticles induce autophagy and endothelial dysfunction via the PI3K/Akt/mTOR signaling pathway. Int J Nanomedicine. 2014; 9: 5131–5141.

El-Hussainy HM et al. 2016; Effects of aluminum oxide (Al2O3) nanoparticles on ECG, myocardial inflammatory cytokines, redox state, and connexin 43 and lipid

profile in rats: possible cardioprotective effect of gallic acid. Can J Physiol Pharmacol. ;94(8):868-78.

Erdely A, et al. 2009. Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers Nano Lett. 9(1):36–43.

Erdely A, et al. 2011. Identification of systemic markers from a pulmonary carbon nanotube exposure. J Occup Environ Med. 2011;53(6 Suppl):S80-6.

Fadeel B et al. There's plenty of room at the forum: potential risks and safety assessment of engineered nanomaterials. Nanotoxicology, June 2007; 1(2): 73-84.

Ge C, et al. Acute pulmonary and moderate cardiovascular responses of spontaneously hypertensive rats after exposure to single-wall carbon nanotubes. Nanotoxicology. 2012;6(5):526-42.

Hong F. Et al. 2015; Toxicological effect of TiO2 nanoparticle-induced myocarditis in mice. Nanoscale Res Lett. 2015; 10: 326.

Kang GS et al. 2011; Long-term inhalation exposure to nickel nanoparticles exacerbated atherosclerosis in a susceptible mouse model. Environmental Health Perspective. 119. 2.

Kreyling WG et al. 2009; Size dependence of the translocation of inhaled iridium and carbon nanoparticleaggregates from the lung of rats to the blood and secondary target organs. Inhal.Toxicol. 21, 55–60.

Li Z, Hulderman T, Salmen R, Chapman R, Leonard SS, Young SH, Shvedova A, Luster MI, Simeonova PP. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. Environ Health Perspect. 2007;115(3):377-82.

Mikkelsen L et al. 2011; Modest effect on plaque progression and vasodilatory function in atherosclerosis-prone mice exposed to nanosized TiO2. Particle and Fibre Toxicology. 1186/1743-8977-8-3.

Mills NL et al. 2006; Do inhaled carbon nanoparticles translocate directly into the circulation in Humans? Am J Respir Crit Care Med. 173(4):426-31

Mills NL et al. 2007; Air pollution and atherothrombosis. Inhal Toxicol. 2007;19 Suppl 1:81-9. Review.

Möller W, et al. 2008; Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. Am J Respir Crit Care Med. 177(4):426–432.

Nemmar A et al. 2007; Enhanced peripheral thrombogenicity after lung inflammation is mediated by platelet-leukocyte activation: role of P-selectin. J Thromb Haemost. 5(6):1217–1226.

Pacurari M et al. 20125;. Cell permeability, migration, and reactive oxygen species induced by multiwalled carbon nanotubes in human microvascular endothelial cells. J Toxicol Environ Health Part A. 75:129–147.

Poulsen SS, Knudsen KB, Jackson P, Weydahl IE, Saber AT, Wallin H, Vogel U. Multi-walled carbon nanotube-physicochemical properties predict the systemic acute

phase response following pulmonary exposure in mice. PLoS One. 2017;12(4):e0174167.

Prawan A, et al. 2005. Molecular basis of heme oxygenase-1 induction: Implications for chemoprevention and chemoprotection. Antioxid Redox Signal. 7(11–12):1688–1703.

Rueda-Romero et al. 2016; Titanium dioxide nanoparticles induce the expression of early and late receptors for adhesion molecules on monocytes. Part Fibre Toxicol. 2015; 13: 36.

Shi H, et al. 2013 Titanium dioxide nanoparticles: a review of current toxicological data. Part Fibre Toxicol. 2013;10:15.

Shvedova AA, et al. 2005; Unusual inflammatory and fibrogenic pulmonary responses to single walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol. 289:L698-L708.

Sun J et al. 2011. Cytotoxicity, permeability, and inflammation of metal oxide nanoparticles in human cardiac microvascular endothelial cells. Cell Biology and Toxicology, Volume 27, Issue 5, pp 333–342.

Upadhyay S et al. 2008. Exposure to ultrafine carbon particles at levels below detectable pulmonary inflammation affects cardiovascular performance in spontaneously hypertensive rats. Part Fibre Toxicol. ;5:19.

Upadhyay S et al. 2014; Ultrafine carbon particle mediated cardiovascular impairment of aged spontaneously hypertensive rats. Part Fibre Toxicol. 2014 Sep 17;11:36.

Vidanapathirana AK et al. 2012; Multi-walled carbon nanotube directed gene and protein expression in cultured human aortic endothelial cells is influenced by suspension medium. Toxicology. 2012 Dec 16; 302(2-3): 114–122.

Walker VG et al. 2009; Potential in vitro effects of carbon nanotubes on human aortic endothelial cells. Toxicology and Applied Pharmacology. 236 (3). 319–328.

4.3. Dermal Toxicity of Nanomaterials

Dermal toxicity to nanoparticles involves the penetration of nanoparticles into skin and the possible systemic effects resulting from such exposure, and the local effect of sensitisation, irritation or corrosion of particles on the skin surface. Skin absorption or dermal penetration of chemicals is a process of diffusion from the outer surface of the skin, through the skin and finally into systemic circulation. In this regard, methods that utilize the entire (intact) skin must be used. The skin consists of three major layers: epidermis, dermis, and subcutis. The outermost layer of the epidermis consists of dead flattened corneocytes and the intercellular space consists of a multilayer of lipids, making up the *stratum corneum*, which is the first barrier to pass for any chemical (WHO, 2006). ENMs have been shown to penetrate through the skin in different ways: (1) *via* hair follicles, (2) intracellularly through corneocytes, or (3) intercellularly around the corneocytes (Baroli et al., 2007; Palmer and DeLouise, 2016). The most common animals used for *in vivo* studies are rats, followed by hairless rats, monkeys, dogs, pigs, guinea pigs, and hairless mice (NEG, 2017). The OECD guideline 427 (OECD, 2004) should be followed for *in vivo* testing of chemicals. During the experiment, care has to be taken to prevent the animal from grooming the exposure site, which would lead to intake of the chemical orally. *In vivo* studies with human volunteers have been performed for several chemicals, but there are certain requirements in order not to risk sensitization of the test subjects to the test substance. ZnO nanoparticles are common in sunscreens; hence, several studies have investigated the penetration of nanoparticles into skin and absorption of nanoparticles through skin in human volunteers. These studies have shown that Zn can be absorbed through the skin after application of sunscreen for 5 consecutive days. However, it could not be concluded whether Zn was absorbed as nanoparticles or as soluble Zn (Gulson et al., 2010, Gulson et al., 2012). Leite-Silva et al. (2013) showed that ZnO nanoparticles were able to penetrate human skin and reach the viable epidermis, but most particles were located in the stratum corneum and in skin furrows.

The standard *in vitro* test for skin penetration of nanoparticles is the OECD guideline 428 using the Franz diffusion cell (OECD, 2004) (Figure 9). The cell consists of two chambers in between which a skin sample is mounted. It is preferable to use *ex vivo* human full thickness skin, but pig skin may also be used. Following exposure, the skin can be studied by using microscopic techniques or by digesting the sample to study the total amount of metal present in the skin. Several metal nanoparticles have been studied using Franz diffusion cells (reviewed in: Filon et al., 2015).

These studies have shown that metal nanoparticles may penetrate though the *stratum corneum*; however, this is mainly true for metal ions and most likely does not correspond to the translocation of the actual nanoparticles.

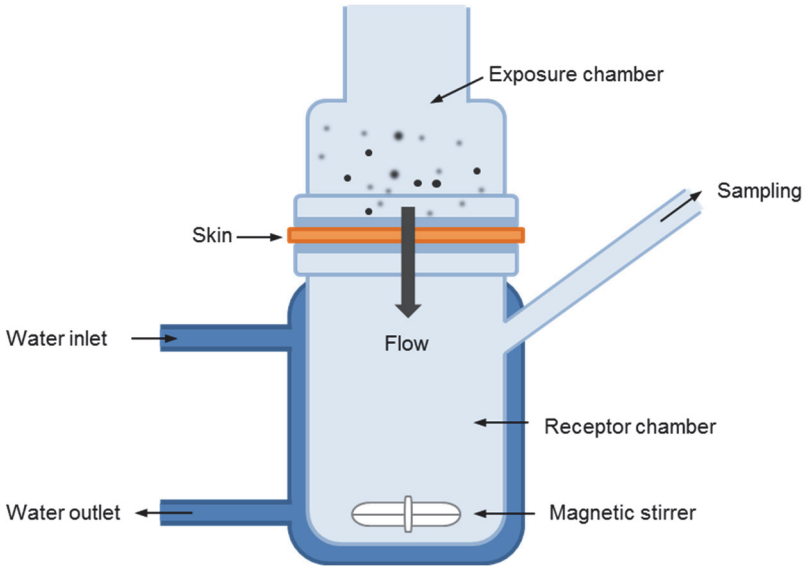


Figure 9. Schematic diagram of a Franz diffusion cell.

Skin irritation / corrosion

Skin irritation refers to the induction of reversible damage of the skin following exposure whereas skin corrosion refers to an irreversible damage. Traditional testing of skin corrosion or irritation has been performed *in vivo* using animals such as rabbits based on the OECD guideline 404 (OECD, 2015). However, the increasing need of reducing test animals have allowed for other *in vitro* methods such as the reconstructed three dimensional human skin (RHE) models; the Epiderm™, EpiSkin™ and skin Ethic™, all used for regulatory purposes (Kim et al., 2016). There are two OECD guidelines on performing irritation and corrosion testing using RHE models (OECD TG431 and OECD TG439). In these, irritancy and corrosiveness is judged on the percentage of cell viability tested with the MTT assay. In a study by Park et al. (2011), using both rabbits and the EpiDerm™ skin model, the irritation and corrosion potential of TiO_2 and polystyrene nanoparticles were evaluated. The results showed that neither type of particle induced irritation or corrosion in either of the two models. In another study using the EpiDerm™ model, iron-, aluminum oxide-, titanium oxide- and silver nanoparticles were tested according to the OECD test guidelines (Kim et al., 2016). The result showed that neither of the tested nanoparticles had a corrosive or irritation effect in the model. These studies confirm the results of a study

using the KeraSkin™ human 3D skin model, in which TiO_2 and ZnO nanoparticles were found to be non-corrosive and non-irritating (Choi et al., 2014).

Sensitization and elicitation

Contact allergy, also known as type IV hypersensitivity, is a result of an immunologic reaction to a substance in contact with the skin. It involves two different phases: induction (sensitization) and elicitation. Methods to measure the induction phase are available. The most common method is the local lymph node assay (LLNA) performed in mice. The LLNA is validated as a method to predict skin sensitization to chemicals (ECVAM, 2008). Many researchers also use the LLNA to sensitize the animals to a test substance, but continues the experiment and challenge the animals 5 days to 2 weeks after sensitization to study the elicitation potential (i.e., the actual contact allergy manifestation) measured both as ear swelling and by immunological parameters such as CD4+ and CD8+ T-cells and cytokine profiles (Vennegaard et al., 2014). Using the LLNA protocol, Park et al. (2011) tested the sensitizing potential of TiO_2 and polystyrene nanoparticles at concentrations ranging from 10 to 1000 µg/mL each. The result showed that neither TiO_2 nor the polystyrene particles exhibited any sensitizing potential (Park et al., 2011). Two other papers have recently been published where sensitization and elicitation has been studied using non-guideline methods in mice to assess several types of nanoparticles (Hirai et al., 2016; Jatana et al., 2017). Hirai et al. (2016) used several mouse models to study silver, nickel, gold and silica nanoparticles. The study showed that only silver and nickel nanoparticles were able to sensitize animal in the presence of an inflammatory stimulus, i.e., lipopolysaccharides, and elicit a response upon challenge. Jatana et al. (2017) used a version of the ear swelling tests in hairless C57BL/6 mice. They showed that when mice were sensitized with a well-known sensitizing substance (DNFB) and then challenged with DNFB or DNFB+nanoparticles, ear swelling was reduced by Au, Ag, Si-nanoparticles, but enhanced by CNTs and TiO_2 nanoparticles (Jantana et al., 2017).

To evaluate the sensitizing potential, the human repeated insult patch test (HRIPT) and the human maximization test (HTM) have been used for several different substances despite the fact that these tests have been criticized for being unethical and for not yielding scientific validity in the results (Basketter et al., 2009). However, the tests are still performed, often outside of Europe, on behalf of industry to evaluate new chemicals. To evaluate the elicitation phase of contact allergy, the diagnostic tool

used in patients with suspected contact allergy is the so-called patch test. However, there are no results of patch tests performed with nanoparticles.

Toxic effects in cultured skin cells

The fact that various ENMs are used in cosmetics and other products that allow contact with skin raises questions regarding their possible toxicity. A common approach for investigating toxicity is to culture human keratinocytes, such HaCaT cells (immortal human keratinocyte cell line) or HEK cells (primary human epidermal keratinocytes isolated from adult skin) and analyze the ability of the NPs to cause toxic effects such as ROS production, induction of inflammation, genotoxicity, and decreased cell viability.

Metal based nanoparticles such as TiO_2, ZnO, Ag and Au nanoparticles are used in cosmetics, e.g., as UV-filters and to achieve antibacterial/anti-inflammatory properties, accelerated wound healing, increase collagen production etc. (Niska et al., 2017). At the same time, studies on cultured skin cells often indicate toxic properties for these nanoparticles. For TiO_2 nanoparticles, one concern is their photocatalytic activity leading to ROS production. The EC Scientific Committee on Consumer Safety (SCCS) concludes in a recent report that it is possible that trace amount of nanoparticles remain embedded in stratum corneum, in hair follicles, and/or sweat glands, over several days after skin application of a product containing TiO_2 nanoparticles. If the nanoparticles have photocatalytic activity, there is a possibility that they may generate ROS upon exposure to sunlight. Furthermore, such ROS production in a close proximity of living cells raises a concern over the possibility of harmful effects (SCCS, 2013). Therefore, SCCS does not recommend the use of nanomaterials that have high photocatalytic activity in dermal formulations (often anatase nanoparticles). Cellular studies have reported on generation of ROS, mitochondrial DNA damage and genotoxic effects in human keratinocytes following exposure of TiO_2 nanoparticles (Niska et al., 2017). It should be noted, however, that for TiO_2 such effects are often observed at relatively high doses (>100 µg/mL), although phototoxicity may be observed also at lower concentration. Horie et al. (2016) showed, for example, that rutile NPs used in sunscreen did not exhibit phototoxic activity following exposure of HaCat cells. Despite the strong phototoxic

“For titanium dioxide (TiO_2) nanoparticles, one concern is their photocatalytic activity leading to ROS production.”

activity of anatase nanoparticles in the cell cultures, no phototoxicity using a 3D skin model was observed. ZnO NPs has shown a wide range of toxic effects in HaCaT cells including impairment of the mitochondrial function, leakage of lactate dehydrogenase (LDH), ROS generation, membrane lipid peroxidation, genotoxicity and apoptosis (Niska et al., 2017). Wang et al. (2013) reported cytotoxicity in HaCaT cells following exposure to ZnO NPs, and the effects were aggravated in the presence of UV-A irradiation. Despite such effects, SCCS concludes in their report that the use of ZnO nanoparticles at a concentration up to 25% as a UV-filter in sunscreens can be considered not to pose a risk of adverse effects in humans after dermal application. Ag nanoparticles have also been studied on skin cells (*in vitro* and *in vivo*). Tian et al. (2007) showed that topical administration of Ag nanoparticles accelerated wound healing in a mouse model. On the other hand, it has been reported that exposure of primary normal human epidermal keratinocytes (NHEK) to Ag nanoparticles decreased their viability as well as their proliferative and migratory potential (Szmyd et al., 2013).

The most well studied carbon-based nanomaterial concerning potential effects on skin cells are the fullerenes, carbon-based, nano-sized hollow structures also known as “buckyballs”. Fullerenes have been reported to possess exceptional antioxidant properties, which has made them an attractive ingredient in many skin care products (Mousavi et al., 2016). Recently, various effects of fullerenes on skin cells were summarized and it was reported that several *in vitro* and some *ex vivo* and *in vivo* studies have shown protective effects of fullerenes against UV-induced skin damages (Mousavi et al., 2016). On the other hand, photocytotoxicity has also been reported (Zhao et al., 2008). It was concluded that most C_{60} derivatives do not alter viability of skin cells in short time and low doses, but viability may be affected over longer periods of exposure or when higher concentrations are used (Mousavi et al., 2016). Sayes et al. (2005) reported, however, an LC50 of C_{60} of 20 ppb, i.e., 0.02 µg/mL, a concentration that must be considered to be very low. In contrast, Monterio-Riviere et al. (2009) reported only minor cytotoxicity of C_{60} in HEK cells. However, it is important to also consider the dispersion protocol that is used since this may affect the toxicity. One study investigated toxicity of a high dose (100 µg/mL) of multi-walled CNTs in keratinocytes and reconstructed human skin. The results showed that water-suspended, non-sonicated CNTs caused only minor effects in keratinocytes, while the addition of hydroxypropylcellulose or Pluronic F108, which improved the dispersion, masked the toxic effects of sonicated CNTs (Vankoningsloo et al., 2010).

References

Baroli, B., Ennas, M. G., Loffredo, F., Isola, M., Pinna, R., and Lopez-Quintela, M. A. (2007). Penetration of metallic nanoparticles in human full-thickness skin. *The Journal of investigative dermatology* **127**(7), 1701-12.

Basketter, D. A. (2009). The human repeated insult patch test in the 21st century: a commentary. *Cutaneous and ocular toxicology* **28**(2), 49-53.

Bastos V, Duarte IF, Santos C, Oliveira H. Genotoxicity of citrate-coated silver nanoparticles to human keratinocytes assessed by the comet assay and cytokinesis blocked micronucleus assay. (2017). *Environ Sci Pollut Res Int*. **24**(5):5039-5048.

Choi, J., Kim, H., Choi, J., Oh, S. M., Park, J., and Park, K. (2014). Skin corrosion and irritation test of sunscreen nanoparticles using reconstructed 3D human skin model. *Environmental health and toxicology* **29**, e2014004.

Gulson, B., McCall, M., Korsch, M., Gomez, L., Casey, P., Oytam, Y., Taylor, A., McCulloch, M., Trotter, J., Kinsley, L.*, et al.* (2010). Small amounts of zinc from zinc oxide particles in sunscreens applied outdoors are absorbed through human skin. *Toxicol. Sci.* **118**(1), 140-149.

Gulson, B., Wong, H., Korsch, M., Gomez, L., Casey, P., McCall, M., McCulloch, M., Trotter, J., Stauber, J., and Greenoak, G. (2012). Comparison of dermal absorption of zinc from different sunscreen formulations and differing UV exposure based on stable isotope tracing. *The Science of the Total Environment* **420**, 313-318.

Hirai, T., Yoshioka, Y., Izumi, N., Ichihashi, K., Handa, T., Nishijima, N., Uemura, E., Sagami, K., Takahashi, H., Yamaguchi, M.*, et al.* (2016). Metal nanoparticles in the presence of lipopolysaccharides trigger the onset of metal allergy in mice. *Nature nanotechnology* **11**(9), 808-16.

Horie M, Sugino S, Kato H, Tabei Y, Nakamura A, Yoshida Y. (2016). Does photocatalytic activity of TiO2 nanoparticles correspond to photo-cytotoxicity? Cellular uptake of TiO2 nanoparticles is important in their photo-cytotoxicity. *Toxicol Mech Methods*. **26**(4):284-94.

Jatana, S., Palmer, B. C., Phelan, S. J., and DeLouise, L. A. (2017). Immunomodulatory Effects of Nanoparticles on Skin Allergy. *Scientific reports* **7**(1), 3979.

Kim, H., Choi, J., Lee, H., Park, J., Yoon, B. I., Jin, S. M., and Park, K. (2016). Skin Corrosion and Irritation Test of Nanoparticles Using Reconstructed Three-Dimensional Human Skin Model, EpiDermTM. *Toxicological research* **32**(4), 311-316.

Larese Filon, F., Mauro, M., Adami, G., Bovenzi, M., and Crosera, M. (2015). Nanoparticles skin absorption: New aspects for a safety profile evaluation. *Regulatory toxicology and pharmacology : RTP* **72**(2), 310-22.

Leite-Silva, V. R., *et al.* (2013). The effect of formulation on the penetration of coated and uncoated zinc oxide nanoparticles into the viable epidermis of human skin in vivo. *European journal of pharmaceutics and biopharmaceutics* **84**(2), 297-308.

Monteiro-Riviere NA, Inman AO, Zhang LW. (2009). Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. *Toxicol Appl Pharmacol*. **234**(2):222-35.

Niska K, Zielinska E, Radomski MW, Inkielewicz-Stepniak I. (2017). Metal nanoparticles in dermatology and cosmetology: Interactions with human skin cells. *Chem Biol Interact*. **16** 30629-9.
OECD (2002). OECD 404: Acute Dermal Irritation/Corrosion In (OECD.
OECD (2004a). OECD 428: guideline for the testing of chemicals. Skin absorption: in vitrotest
OECD (2014). OECD 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method. In (
OECD (2015). OECD 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, OECD Guidelines for the Testing of Chemicals, Section 4 Health Effects.
OECD (2004b). Test No. 427: Skin Absorption: In Vivo Method. In (doi: 10.1787/9789264071063-en. OECD.
Palmer, B. C., and DeLouise, L. A. (2016). Nanoparticle-Enabled Transdermal Drug Delivery Systems for Enhanced Dose Control and Tissue Targeting. *Molecules (Basel, Switzerland)* **21**(12).
Park, Y. H., Jeong, S. H., Yi, S. M., Choi, B. H., Kim, Y. R., Kim, I. K., Kim, M. K., and Son, S. W. (2011). Analysis for the potential of polystyrene and TiO2 nanoparticles to induce skin irritation, phototoxicity, and sensitization. *Toxicology in vitro* **25**(8), 1863-9.
SCCS (Scientific Committee on Consumer Safety), (2014). Opinion on Titanium Dioxide (nano form).
SCCS (Scientific Committee on Consumer Safety), (2011). Opinion on Zinc oxide (nano form), SCCS/1489/12.
Sayes CM, Gobin AM, Ausman KD, Mendez J, West JL, Colvin VL. (2005). Nano-C60 cytotoxicity is due to lipid peroxidation. *Biomaterials*. **26**(36):7587-95.
Szmyd R, Goralczyk AG, Skalniak L, Cierniak A, Lipert B, Filon FL, Crosera M, Borowczyk J, Laczna E, Drukala J, Klein A, Jura J. (2013) Effect of silver nanoparticles on human primary keratinocytes. *Biol Chem*. **394**(1):113-23.
Theraputic Goods Administration, T. (2016). Literature review on the safety of titanium dioxide and zinc oxide nanoparticles in sunscreens. Scientific review report.
Tian J, Wong KK, Ho CM, Lok CN, Yu WY, Che CM, Chiu JF, Tam PK. Topical delivery of silver nanoparticles promotes wound healing. ChemMedChem. 2007;2(1):129-36.
Vankoningsloo S, Piret JP, Saout C, Noel F, Mejia J, Zouboulis CC, Delhalle J, Lucas S, Toussaint O. (2010). Cytotoxicity of multi-walled carbon nanotubes in three skin cellular models: effects of sonication, dispersive agents and corneous layer of reconstructed epidermis. *Nanotoxicology*. **4**(1):84-97.
Vennegaard, M. T., Dyring-Andersen, B., Skov, L., Nielsen, M. M., Schmidt, J. D., Bzorek, M., Poulsen, S. S., Thomsen, A. R., Woetmann, A., Thyssen, J. P.*, et al.* (2014). Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact dermatitis* **71**(4), 224-32.
Wang CC, Wang S, Xia Q, He W, Yin JJ, Fu PP, Li JH. (2013). Phototoxicity of zinc oxide nanoparticles in HaCaT keratinocytes-generation of oxidative DNA damage during UVA and visible light irradiation. *J Nanosci Nanotechnol*. **13**(6):3880-8.
WHO (2014). Environmental Health Criteria 242. Dermal exposure. *IPCS* http://www.who.int/ipcs/publications/ehc/ehc_242.pdf.

Zhao B, He YY, Bilski PJ, Chignell CF. (2008) Pristine (C60) and hydroxylated [C60(OH)24] fullerene phototoxicity towards HaCaT keratinocytes: type I vs type II mechanisms. *Chem Res Toxicol*. **21**(5):1056-63.

4.4. Developmental Toxicity of Nanomaterials

It is broadly accepted that the fetus may be more sensitive to chemical exposures than the adult organism. However, the available information on potential developmental toxicity of ENMs is limited and remains insufficient as a basis for risk assessment for pregnant women and their children (Hougaard and Campagnolo, 2012). Indeed, it is important to understand whether ENMs are translocated from the port of entry (mostly the lungs) to the fetus through the systemic circulation and whether they are capable of crossing the placental barrier (Klein et al., 2012; Pietroiusti, et al., 2013). On the other hand, during pregnancy the induced inflammation and oxidative stress elicted by ENMs may have detrimental consequences on fetal development (Hougaard et al., 2010; Pietroiusti et al., 2013). Moreover, inflammation in the lung may potentially result in the disruption of the maternal hormonal balance during pregnancy and may lead to abnormal growth of the fetus (Karsch et al., 2002). Clearly, more studies are needed to understand the potential for developmental toxicity of ENMs.

Zebra fish have been used as a model in several studies to explore potential developmental effects. Hence, ZnO nanoparticles were found to affect genes related to inflammation and the immune system, resulting in yolk-sac edema and pericardia edema in embryonic/larval developmental stages in zebra fish (Choi et al., 2016). TiO_2 nanoparticles were shown to downregulate the expression of many genes that regulate the circadian rhythm, kinase-related activities and immune response (Jovanović et al., 2011). In contrast, Zhu et al. (2008) found that TiO_2 nanoparticles displayed no toxic effects in zebra fish up to a concentration of 500 µg/mL. Exposure of zebra fish to multi-walled CNTs induced hatching delays and increased mortality rates in treated embryos (Asharani et al., 2008). Kong et al. (2014) have shown induced reproductive toxicity in rats exposed to Ni nanoparticles by gavage. Jo et al. (2013) exposed rats to ZnO nanoparticles (500 mg/kg) beginning at 2 weeks before mating to postnatal day 4, and concluded that ZnO nanoparticle exposure before and during pregnancy and lactation could pose health risks to pregnant mothers and their fetus. Pietroiusti et al. (2011) demonstrated that intravenous administration of both pristine and oxidized single-walled carbon nanotubes (SWCNTs) (Figure 10) in pregnant CD-1 mice was found to induce morphological abnormalities in the fetuses as observed by deformities in the abdominal wall or head, retarded development of the limbs and snout. In contrast, toxic responses were not observed following administration of multi-walled carbon nanotubes (MWCNTs) by gavage to pregnant Sprague-Dawley rats (Lim et al., 2011). These observations

indicate that nanomaterial-induced developmental toxicity is highly dependent on dose, route of exposure and species. The effects of ENMs on reproductivity and fetal development need to be explored in more detail, and multi-generation studies with short-term and long-term exposures to different ENMs at different phases of the reproductive cycle (before and during pregnancy, during gestation and lactation period) need to be considered.

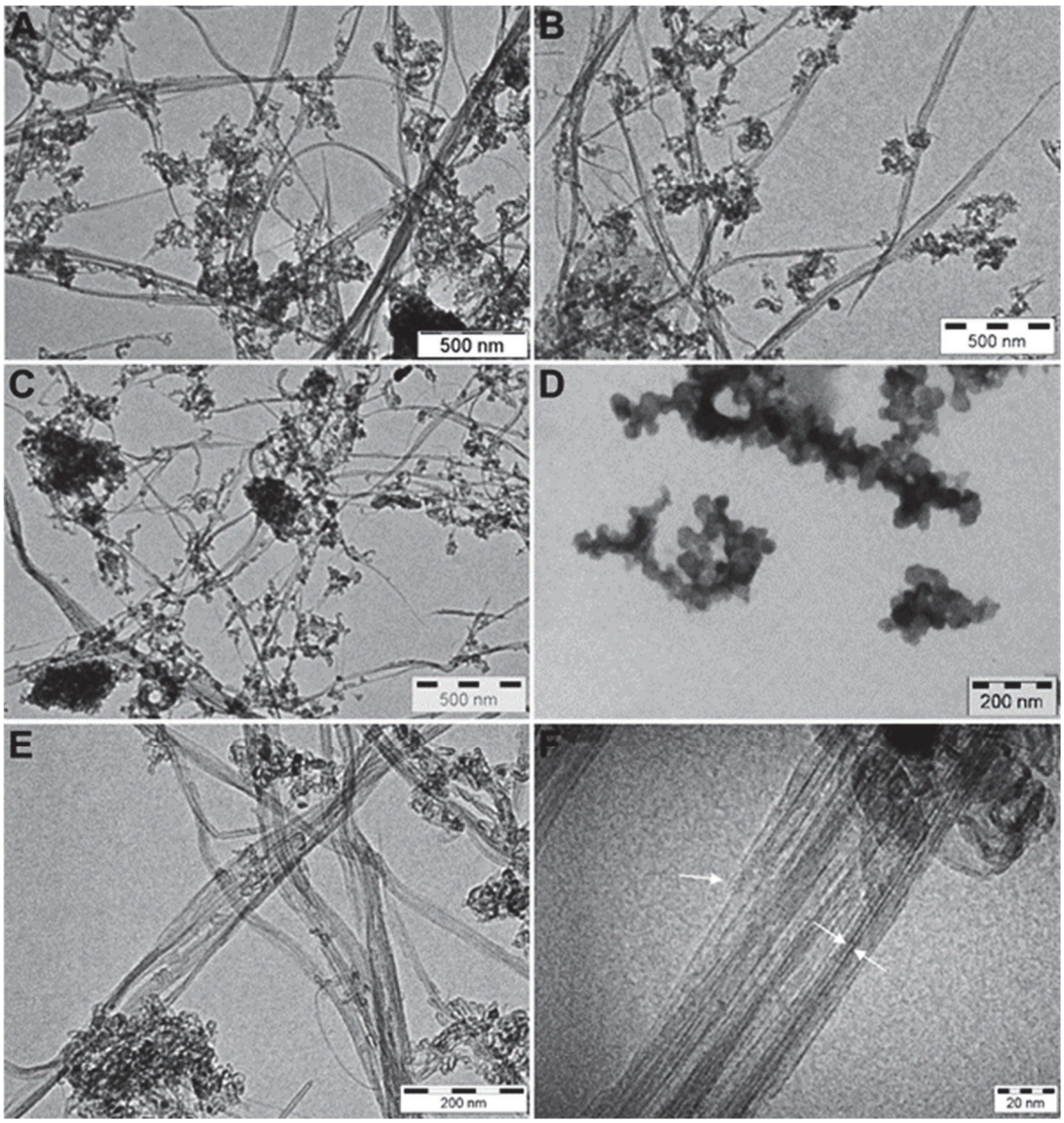


Figure 10. TEM images of (A) pristine SWCNTs, (B) oxidized SWCNTs, (C) 'ultraoxidized' SWCNTs, and (D) nano-sized carbon black. SWCNTs appear organized in bundles of different length. A higher abundance of short bundles is observed in 'ultraoxidized' SWCNTs. High-magnification images for oxidized SWCNTs are shown in E and F. In panel F, individual SWCNTs are visible within the bundles (arrows). These materials have been studied for their potential impact on pregnant mice following i.v. injection, as described in Pietroiusti et al. (2011). A higher percentage of fetal malformations was observed in female mice exposed to 'ultraoxidized' SWCNTs.

References

Asharani, P, Serina NG, Nurmawati MH, Wu YL, Gong Z, Valiyaveettil S. 2008; Impact of multi-walled carbon nanotubes on aquatic species. J. Nanosci. Nanotechnol. 2008, 8, 3603–3609.

Choi JS, Kim RO, Yoon S, Kim WK. 2016; Developmental toxicity of zinc oxide nanoparticles to zebrafish (Danio rerio): a transcriptomic analysis. PLoS One. 11(8):e0160763.

Hougaard KS. 2015, A perspective on the developmental toxicity of inhaled nanoparticles. J. Environ. Sci. Health A 2008, 43, 278–284.

Jo E, Seo G, Kwon JT, Lee M, Lee Bc, Eom I, Kim P, Choi K. 2013; Exposure to zinc oxide nanoparticles affects reproductive development and biodistribution in offspring rats. J Toxicol Sci. 2013;38(4):525-30.

Jovanović, B, Ji T, Palić D. 2011. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. Ecotoxicol. Environ. Saf. 2011, 74, 1518–1525.

K.S. Hougaard, L. Campagnolo. Reproductive toxicity of engineered nanoparticles. In: B. Fadeel, A. Pietroiusti, A.A. Shvedova (Eds.), *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health*. Elsevier (2012), pp. 225-248.

Karsch FJ, Battaglia DF, Breen KM, Debus N, Harris TG. 2002; Mechanisms for ovarian cycle disruption by immune/inflammatory stress. Stress, 5 (2) (2002), pp. 101-112.

Klein CL. et al. 2012. Identification of inhaled nanomaterials: making use of short-term inhalation studies. Arch Toxicol, 86 (7) (2012), pp. 1137-1151.

Kong L, Tang M, Zhang T, Wang D, Hu K, Lu W, Wei C, Liang G, Pu Y. 2014; Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats. Int. J. Mol. Sci. 15(11), 21253-21269.

Lim JH, Kim SH, Shin IS, Park NH, Moon C, Kang SS, Kim SH, Park SC, Kim JC. 2011. Maternal exposure to multi-wall carbon nanotubes does not induce embryo-fetal developmental toxicity in rats. Birth Defects Res.B, 92, 69–76.

Pietroiusti A, Campagnolo L, Fadeel B. 2013. Interactions of engineered nanoparticles with organs protected by internal biological barriers. Small, 9 (9–10). 1557-1572.

Pietroiusti A. Massimiani M, Fenoglio I, Colonna M, Valentini F, Palleschi G, Camaioni A, Magrini A, Siracusa G, Bergamaschi A, Sgambato A, Campagnolo L. 2011, Low doses of pristine and oxidized single wall carbon nanotubes affect mammalian embryonic development. ACS Nano 5, 4624–4633.

Zhu, X, Zhu L, Duan Z, Qi R, Li Y, Lang Y.. 2008; Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (Danio rerio) early developmental stage. J Environ Sci Health A Tox Hazard Subst Environ Eng43(3):278-84.

4.5. Immunotoxicity of Nanomaterials

The immune system is an intricate system of cells and signaling molecules that communicate with each other to maintain homeostasis and provide immediate responses to invading pathogens and other foreign bodies. The immune system comprises of the innate and acquired immune system. The major components of the innate immune system are the professional phagocytic cells such as macrophages, along with neutrophils, mast cells, eosinophils and basophils (Farrera and Fadeel, 2015; Boraschi et al., 2017). Natural killer (NK) cells also belong to the innate immune system and are specialized in warding of cancerous cells. The key players of the adaptive immune system are the B and T cells while dendritic cells (DCs) act as a bridge between the innate and adaptive immune system (Figure 11). Here we will focus on interactions of ENMs with the innate immune system.

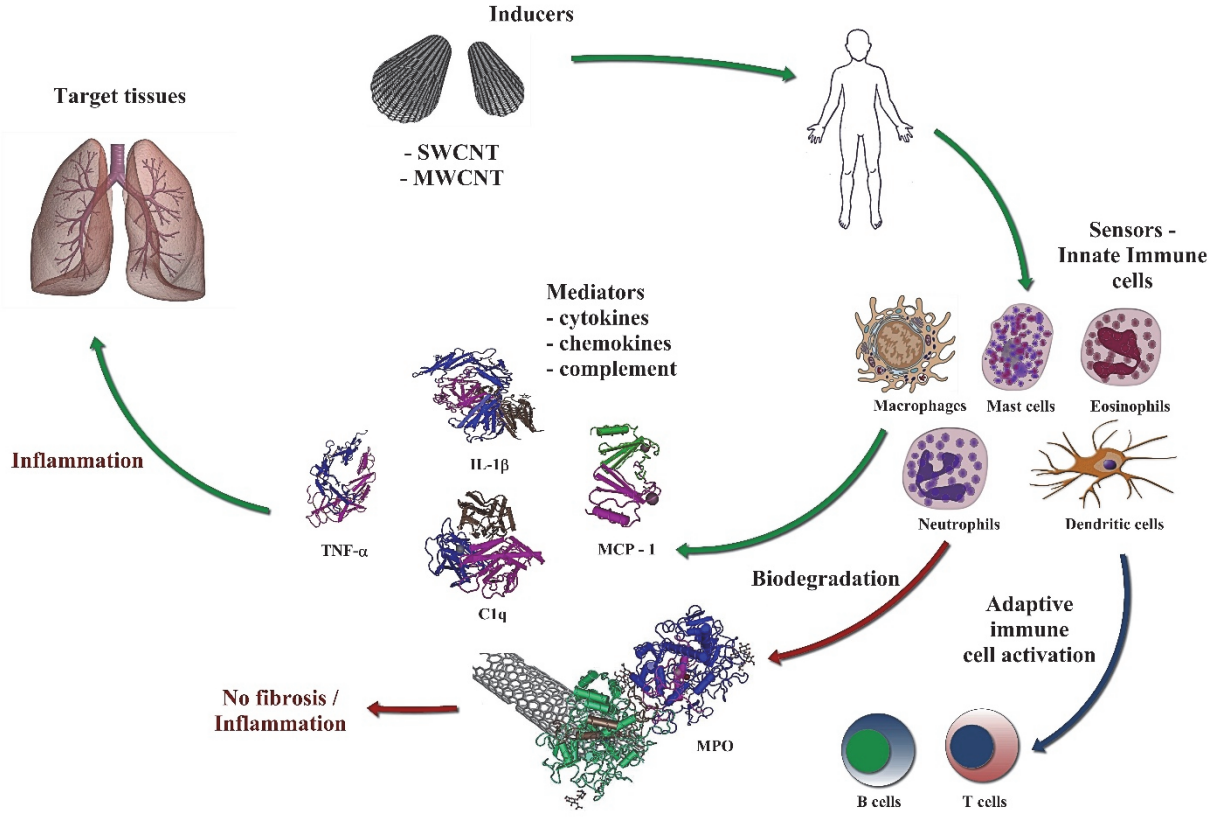


Figure 11. Reciprocal interactions of nanomaterials and the immune system. Exposure to so-called *inducers* (here, single- and multi-walled CNTs) initiates a multifactorial response from the *sensors* (i.e., innate immune cells). These cells produce soluble *mediators* (cytokines, chemokines, and complement factors; e.g., IL-1β, TNF-α, C1q, MCP-1, and many others) leading to inflammation in target tissues, such as the lungs. Several studies have shown that inflammatory cells (neutrophils, eosinophils, macrophages) are capable of biodegradation of CNTs. From: Bhattacharya et al., 2013.

Sensing of nanomaterials by the immune system

Macrophages are professional phagocytic cells belonging to the reticuloendothelial system with both proteolytic and catabolic capabilities, and they are involved in phagocytosis of foreign bodies including pathogens as well as the clearance of apoptotic cell debris. Macrophages are known by different names depending on their location in the body, such as, alveolar macrophages in lungs, osteoclasts in bone, microglia in the brain, and Kupffer cells in the liver (Galli et al., 2011). Pathogens are detected by macrophages using cell surface receptors such as Toll-like receptors (TLRs), and the engagement of these receptors leads to the release of inflammatory mediators (Medzhitov, 2008; *idem*, 2010). Macrophage responses to ENMs have been investigated in numerous studies, though it is pertinent to note that in many cases, macrophage-like cancer cell lines and not primary macrophages have been used; it is also important to note that the activation status of the macrophage may affect interactions with ENMs and the consequences of such interactions – not all macrophages are alike. The bio-corona on the surface of nanomaterials may conceivably affect the subsequent interaction with macrophages (Mortimer et al., 2014; Cheng et al., 2015). However, the presence of a bio-corona does not necessarily promote cellular uptake of nanoparticles, and the specific composition of the corona may come into play (Vogt et al., 2015). Balasubramanian et al. (2013) observed in a study using gold nanoparticles (7 and 20 nm) that the primary particle size is important for macrophage mediated clearance in the lungs following inhalation exposure. Earlier work performed on TiO_2 nanoparticles (20 nm) provided evidence for a sporadic and rather unspecific uptake of particles by lung macrophages 24 h after their deposition and, hence, for an insufficient role of this key clearance mechanism in peripheral lungs (Geiser et al., 2008).

Macrophage activation status may determine the propensity for uptake of nanoparticles (Gallud et al., 2017; MacParland et al., 2017). Scavenger receptors have been suggested to be involved in the uptake of some types of nanoparticles though other macrophage receptors may also play a role (Gallud et al., 2017). In a recent study, Tsugita et al. (2017) applied functional expression cloning and identified the class B scavenger receptor SR-B1 as a *bona fide* receptor for silica. Thus, through an extracellular α-helix, both mouse and human SR-B1 specifically recognized amorphous and crystalline silica, but not TiO_2 nanoparticles, latex nanoparticles, or monosodium urate crystals, even though all these

particles displayed a negative surface potential. Furthermore, SR-B1 was found to be involved in silica-induced pulmonary inflammation in mice (Tsugita et al., 2017). Jones et al. (2013) found that mouse strains that are prone to Th1 immune responses clear nanoparticles at a slower rate than Th2-prone mice. Treating macrophages from Th1 strains with cytokines to differentiate them into M2 macrophages increased the amount of particle uptake. Conversely, treating macrophages from Th2 strains with cytokines to differentiate them into M1 macrophages decreased their particle uptake. The authors suggested that the immune activation status is likely to affect nanoparticle clearance in humans too. Scoville et al. (2017) exposed 25 different inbred mouse strains to citrate-coated Ag nanoparticles and measured neutrophils in bronchoalveolar lavage (BAL) fluid as a marker of lung inflammation, and used genome-wide association mapping to identify candidate genes are associated with Ag nanoparticle-induced lung inflammation. The authors identified three candidate genes for which mRNA levels inversely correlated with Ag nanoparticle-induced lung inflammation in mice. These studies are important as they point to underlying genetic differences in susceptibility to nanomaterial-induced inflammatory responses, and also serve as a reminder that a model is only a model.

Pattern recognition receptors (PRRs) localized at the cell surface, or inside the cell, are an important feature of the immune system as these receptors enable cells at the front line of host defense, such as macrophages and epithelial cells, to detect and respond to the presence of so-called danger- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively). PAMPs include bacterial cell wall components (eg., lipopolysaccharide, and peptidoglycan) or viral DNA/RNA, as well as fungal glucans. DAMPs, on the other hand, are endogenous stress signals (also known as alarmins) that are released from dying cells, and their recognition by immune cells *via* different PRRs results in immune cell maturation and the production of pro-inflammatory cytokines (Farrera and Fadeel, 2015). It has been hypothesized that nanomaterial surfaces may be recognized as NAMPs (nanomaterial-associated molecular patterns) by the immune system (Fadeel, 2012). Indeed, recent studies conducted at Karolinska Institutet in Stockholm have provided evidence that single-walled CNTs may interact with Toll-like receptors (TLRs) on the surface of macrophages leading to transcriptional upregulation of several chemokine-encoding genes (Mukherjee et al., 2018a). Graphene oxide, on the other hand, did not show any effect on chemokines. Care was taken to ensure that the SWCNTs were not endotoxin contaminated. Interestingly, TLR signaling occurred both in the presence and absence of

serum proteins suggesting that the effect was not related to the bio-corona of proteins adsorbed on the CNTs. Molecular modeling suggested that CNTs could interact directly with TLRs. Collectively, the authors proposed that SWCNTs are ‘sensed’ as pathogens by PRRs (Mukherjee et al., 2018a).

Neutrophils are short-lived granular leukocytes or granulocytes possessing a nucleus with three or more lobes (i.e., polymorphonuclear cells). Neutrophils are normally found circulating in the blood stream, but they can extravasate and enter into tissues in response to a foreign stimulus, and are considered as a hallmark of acute inflammation. Neutrophils engulf and kill pathogens including bacteria through the generation of reactive oxygen species (ROS), or by using degradative enzymes such as elastase. Neutrophils are also involved in non-infectious (sterile) inflammation. Exposure to a variety of metal-based or carbonaceous nanomaterials has been found to induce neutrophilic inflammation (see, for instance, Morimoto et al., 2012; Poulsen et al., 2016; Larsen et al., 2016; Ramadi et al., 2016). However, despite numerous studies showing the infiltration of neutrophils upon pulmonary exposure to nanoparticles, it can be argued that neutrophils are somewhat neglected cells in nanotoxicology (and in nanomedicine) as there are relatively few studies on the direct interactions of ENMs with neutrophils while there is a great preponderance of studies on macrophages. However, TiO_2 nanoparticles have been shown to activate neutrophils (Gonçalves et al., 2010) leading to degranulation of the cells (Babin et al., 2013). Moreover, both TiO_2 and ZnO nanoparticles were shown to inhibit neutrophil cell death (apoptosis) (Gonçalves et al., 2010; Goncalves and Girard, 2014). Gold nanoparticles, on the other hand, triggered neutrophil apoptosis (Noël et al., 2016). Small, carbon-based nanoparticles (nanodiamonds) were recently shown to trigger the formation of so-called neutrophil-extracellular traps (NETs) *in vitro* and *in vivo* when administered at high doses leading to a self-resolving inflammation in mice (Muñoz et al., 2016). NETs are generated in response to exogenous (bacteria, fungi) as well as endogenous (cholesterol, urate crystals) stimuli allowing neutrophils to ‘capture’ (and destroy) foreign bodies extracellularly (Brinkmann and Zychlinsky, 2007). However, excessive formation of NETs may lead to pathology (reviewed in: Papayannopoulos, 2018). The authors of the latter nanodiamond study speculated that the containment of nanoparticles in aggregates of NETs orchestrates the resolution of inflammation (Muñoz et al., 2016). In more recent studies, graphene oxide (GO) sheets were found to trigger NETs through a lipid peroxidation-dependent mechanism and this resulted in the ‘capture’ and degradation

of GO in NETs (Mukherjee et al., 2018b; Mukherjee et al., 2018c). Neutrophils can also enzymatically 'digest' at least some forms of CNTs (discussed below). Thus, it appears that neutrophils are capable of responding to pathogens and ENMs in a similar manner leading to destruction of the offending agent (Farrera and Fadeel, 2015). Eosinophils and mast cells are also components of the innate immune system. Both cells are involved in allergic responses. Carbon nanofibers and nanotubes both increased IgE levels in mouse models of allergy, but CNTs and not the carbon nanofibers promoted eosinophilic lung inflammation (Nygard et al., 2013), suggesting that nanotubes are particularly potent in promoting allergic responses. Rydman et al. (2014) reported that short-term inhalation of rigid, rod-like CNTs, but not flexible, tangled CNTs, induced airway inflammation in mice resembling allergic inflammation with marked eosinophilia. In addition, mast cells were found to partially regulate the inflammation caused by rod-like CNTs. Katwa et al. (2012) demonstrated the involvement of mast cells in pulmonary and cardiovascular responses to multi-walled CNTs. Johnson et al. (2017) sought to identify genetic factors playing a role in silver nanoparticle-induced mast cell degranulation, and discovered novel genes not previously known to play a role in particle-mediated mast cell activation. Activation of mast cells can lead to the production of histamine, leukotrienes, proteases, cytokines, chemokines, and other substances that cause immediate airway inflammation, leading to asthma symptoms, and such studies may lead to the identification of novel therapeutic targets in mast cell-linked disorders.

Degradation of nanomaterials by innate immune cells

The fibre-like aspects of CNTs have been compared to asbestos, raising concerns that widespread use of CNTs may lead to mesothelioma, a malignant cancer caused by asbestos exposure. Pathogenic asbestos fibres are biopersistent. However, studies published during the past 10 years have provided evidence that certain forms of CNTs are susceptible to biodegradation. Hence, oxidized, but not pristine single-walled CNTs were shown to undergo degradation upon *in vitro* incubation with horseradish peroxidase (HRP) and low concentrations of H_2O_2 (Allen et al., 2008; *idem*, 2009). Kagan et al. (2010) reported for the first time that neutrophil myeloperoxidase (MPO) released from degranulating human neutrophils is capable of efficiently degrading oxidized SWCNTs, and in a subsequent study, *in vivo* evidence for the MPO-dependent clearance of SWCNTs from the lungs of exposed animals was provided (Shvedova et al., 2012). However, clearance of CNTs from the lungs may also occur through translocation to extra-pulmonary sites (Jacobssen et al., 2017).

Furthermore, both SWCNTs and GO undergo MPO-mediated degradation in purified NETs (Farrera et al., 2014; Mukherjee et al., 2018b). Importantly, the MPO-generated degradation products of GO were shown to be non-genotoxic for human lung cells (Mukherjee et al., 2018b). Eosinophils have also been observed to biodegrade oxidized SWNCTs through peroxidase-based mechanisms (Andon et al., 2013), and lactoperoxidase (LPO)-mediated degradation of CNTs has been reported (Bhattacharya et al., 2015). LPO is a constitutively secreted enzyme, while MPO and EPO are stored in granules and are only released upon activation of neutrophils and eosinophils, respectively. Therefore, the LPO system may confer a basal defense of the airways not only against pathogens (Gerson et al., 2000), but also against nanomaterials. On the other hand, studies have shown that multi-walled CNTs are not as efficiently degraded as SWCNTs (Russier et al., 2011). Furthermore, graphene is not as readily degraded by neutrophils as GO (Kurapati et al., 2018). Degradability or biopersistance is very likely an important determinant of the long-term impact of carbonaceous materials including CNTs.

Profiling of inflammogenic potential of nanomaterials

According to the EU-funded NANOREG project, immunotoxicity is one of the key elements of a nano-specific approach to risk assessment (Dekkers et al., 2016). Recent studies performed in NANOREG project using macrophage-like THP.1 cells as a model have shown that ENMs can be segregated into two distinct groups characterized by activation and deactivation, respectively, of specific nuclear receptor pathways (Bhattacharya et al., 2017). This represents a first step towards grouping of ENMs on the basis of *in vitro* 'inflammogenic' potential. However, different types of inflammation imply different hazards, and *in vitro* testing may not readily distinguish between different patterns of inflammation. Cho et al. (2010) investigated the inflammation potencies of a panel of panel of well-characterized metal and metal oxide nanoparticles (CeO_2, TiO_2, SiO_2, ZnO, NiO, CuO nanoparticles) *versus* carbon black nanoparticles following instillation into lungs of rats. The exposures were carried out at equal-surface-area doses. Only CeO_2, NiO, ZnO, and CuO nanoparticles were inflammogenic at the high doses used. Interestingly, each of these induced a unique inflammatory 'footprint' both acutely (at 24 h) and chronically (4 weeks), with neutrophilic, neutrophilic/lymphocytic, eosinophilic/fibrotic/granulomatous, and fibrotic/granulomatous inflammation being caused, respectively, by CeO_2, NiO, ZnO, and CuO nanoparticles. The study underscores that ENMs cannot be viewed as a

single hazard entity and that risk assessment should be performed on a case-by-case basis until that time when nanoparticles can be reliably grouped.

Several studies have shown that ENMs triggers activation of the so-called *inflammasome* complex in macrophages (Bhattacharya et al., 2013). Several members of the NOD-like receptor (NLR) family, a subset of cytoplasmic PRRs, are able to sense PAMPs and DAMPs and subsequently induce the assembly of a multi-protein signalling platform called the inflammasome (Broz and Dixit, 2016). The inflammasome serves as an activation platform for caspase-1, a central mediator of innate immunity. Active caspase-1, in turn, promotes the maturation and release of interleukin-1β (IL-1β). Dostert et al. (2008) were the first to show that asbestos fibers and crystalline silica trigger activation of the NLRP3 [nucleotide-binding domain, leucine-rich family (NLR), pyrin-containing 3] inflammasome, and several other studies have subsequently shown that the NLRP3 inflammasome also responds to a range of different ENMs (Farrera and Fadeel, 2015; Sayan and Mossman, 2016). The mechanism of activation of the NLRP3 inflammasome has been the subject of much attention, and (ROS production, lysosomal release of cathepsin B, and a drop in intracellular potassium levels have all been implicated in this process. Xia and co-workers reported that rare earth oxide nanoparticles triggered inflammasome activation through a biotransformation process within lysosomes resulting in organelle damage, with release of cathepsin B (Li et al., 2014a). In a subsequent study, Li et al. (2014b) found that rare earth oxide nanoparticles interfered with autophagosome fusion with lysosomes thereby disrupting the regulation of activated NLRP3 complexes, leading, in turn, to elevated IL-1β production. Together, these findings serve to highlight the molecular mechanism of metal oxide-induced inflammasome activation. However, whether this is to be considered a ‘novel’ mechanism is debatable, as the lysosomal pathway in macrophages is also engaged in response to pathogens. Hence, the pathway may be novel for nanotoxicologists, but not to the immune system.

Concluding remarks

Cells of the innate immune system behave as sentinels, monitoring the body for foreign intruders including pathogens and nanoparticles, and orchestrating inflammatory responses, as needed. It should be noted, however, that the purpose of the inflammatory response is to remove or sequester the offending agent, to allow the organism to adapt, and

“The purpose of the inflammatory response is to remove or sequester the offending agent.”

ultimately, to restore functionality to the tissues (Medzhitov, 2008). If the process becomes chronic, the adaptive changes may become detrimental. Thus, it is important, as we consider the inflammatory potential of ENMs, to distinguish between transient, protective responses and chronic, maladaptive ones. Unbridled inflammation and fibrosis may lead to disease.

References

Allen BL, Kichambare PD, Gou P, Vlasova II, Kapralov AA, Konduru N, Kagan VE, Star A. Biodegradation of single-walled carbon nanotubes through enzymatic catalysis. Nano Lett. 2008;8(11):3899-903.

Allen BL, Kotchey GP, Chen Y, Yanamala NV, Klein-Seetharaman J, Kagan VE, Star A. Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes. J Am Chem Soc. 2009;131(47):17194-205.

Andón FT, Kapralov AA, Yanamala N, Feng W, Baygan A, Chambers BJ, Hultenby K, Ye F, Toprak MS, Brandner BD, Fornara A, Klein-Seetharaman J, Kotchey GP, Star A, Shvedova AA, Fadeel B, Kagan VE. Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. Small. 2013;9(16):2721-9, 2720.

Babin K, Antoine F, Goncalves DM, Girard D. TiO2, CeO2 and ZnO nanoparticles and modulation of the degranulation process in human neutrophils. Toxicol Lett. 2013;221(1):57-63.

Balasubramanian SK, Poh K-W, Ong C-N, Kreyling WG, Ong W-Y, Yu LE, (2013). The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. Biomaterials, 34, 5439-5452.

Bhattacharya K, Andón FT, El-Sayed R, Fadeel B. Mechanisms of carbon nanotube-induced toxicity: focus on pulmonary inflammation. Adv Drug Deliv Rev. 2013;65(15):2087-97.

Bhattacharya K, El-Sayed R, Andón FT, Mukherjee SP, Gregory J, Li H, Zhao Y, Seo W, Fornara A, Brandner B, Toprak MS, Leifer K, Star A, Fadeel B. Lactoperoxidase-mediated degradation of single-walled carbon nanotubes in the presence of pulmonary surfactant. Carbon. 2015;91:506-517.

Bhattacharya K, Kilic G, Costa PM, Fadeel B. Cytotoxicity screening and cytokine profiling of nineteen nanomaterials enables hazard ranking and grouping based on inflammogenic potential. Nanotoxicology. 2017;11(6):809-826.

Boraschi D, Fadeel B, Duschl A. Immune system, in: *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health*, Eds. B. Fadeel, A. Pietroiusti, A. Shvedova. Elsevier-Academic Press, Amsterdam, 2017 (2nd edition), pp. 313-337.

Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol. 2007;5(8):577-82.

Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol. 2016;16(7):407-20.

Cheng X, Tian X, Wu A, Li J, Tian J, Chong Y, Chai Z, Zhao Y, Chen C, Ge C. Protein corona influences cellular uptake of gold nanoparticles by phagocytic and nonphagocytic cells in a size-dependent manner. ACS Appl Mater Interfaces. 2015;7(37):20568-75.

Dekkers S, Oomen AG, Bleeker EA, Vandebriel RJ, Micheletti C, Cabellos J, Janer G, Fuentes N, Vázquez-Campos S, Borges T, Silva MJ, Prina-Mello A, Movia D, Nesslany F, Ribeiro AR, Leite PE, Groenewold M, Cassee FR, Sips AJ, Dijkzeul A, van Teunenbroek T, Wijnhoven SW. Towards a nanospecific approach for risk assessment. Regul Toxicol Pharmacol. 2016;80:46-59.

Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science. 2008;320(5876):674-7.

Fadeel B. Clear and present danger? Engineered nanoparticles and the immune system. Swiss Med Wkly. 2012;142:w13609.

Farrera C, Bhattacharya K, Lazzaretto B, Andón FT, Hultenby K, Kotchey GP, Star A, Fadeel B. Extracellular entrapment and degradation of single-walled carbon nanotubes. Nanoscale. 2014;6(12):6974-83.

Farrera C, Fadeel B. It takes two to tango: understanding the interactions between engineered nanomaterials and the immune system. Eur J Pharm Biopharm. 2015;95(Pt A):3-12.

Galli S.J., Borregaard N., Wynn T.A., Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils, Nat Immunol, 12 (2011) 1035-1044.

Gallud A, Bondarenko O, Feliu N, Kupferschmidt N, Atluri R, Garcia-Bennett A, Fadeel B. Macrophage activation status determines the internalization of mesoporous silica particles: exploring the role of different pattern recognition receptors. Biomaterials. 2017;121:28-40.

Geiser M, Casaulta M, Kupferschmid B, Schulz H, Semmler-Behnke M, Kreyling W. The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. Am J Respir Cell Mol Biol. 2008;38(3):371-6.

Gerson C, Sabater J, Scuri M, Torbati A, Coffey R, Abraham JW, Lauredo I, Forteza R, Wanner A, Salathe M, Abraham WM, Conner GE. The lactoperoxidase system functions in bacterial clearance of airways. Am J Respir Cell Mol Biol. 2000;22(6):665-71.

Gonçalves DM, Chiasson S, Girard D. Activation of human neutrophils by titanium dioxide (TiO2) nanoparticles. Toxicol In Vitro. 2010;24(3):1002-8.

Goncalves DM, Girard D. Zinc oxide nanoparticles delay human neutrophil apoptosis by a de novo protein synthesis-dependent and reactive oxygen species-independent mechanism. Toxicol In Vitro. 2014;28(5):926-31.

MacParland SA, Tsoi KM, Ouyang B, Ma XZ, Manuel J, Fawaz A, Ostrowski MA, Alman BA, Zilman A, Chan WC, McGilvray ID. Phenotype determines nanoparticle uptake by human macrophages from liver and blood. ACS Nano. 2017;11(3):2428-2443.

Mukherjee SP, Bondarenko O, Kohonen P, Andón FT, Brzicová T, Gessner I, Mathur S, Bottini M, Calligari P, Stella L, Kisin E, Shvedova A, Autio R, Salminen-Mankonen H, Lahesmaa R, Fadeel B. Macrophage sensing of single-walled carbon nanotubes via Toll-like receptors. Sci Rep. 2018a;8(1):1115.

Mukherjee SP, Gliga AR, Lazzaretto B, Brandner B, Fielden M, Vogt C, Newman L, Rodrigues AF, Shao W, Fournier PM, Toprak MS, Star A, Kostarelos K, Bhattacharya K, Fadeel B. Graphene oxide is degraded by neutrophils and the degradation products are non-genotoxic. Nanoscale. 2018b;10(3):1180-1188.

Mukherjee SP, Lazzaretto B, Hultenby K, Newman L, Rodrigues AF, Lozano N, Kostarelos K, Malmberg P, Fadeel B. Graphene oxide elicits membrane lipid changes and neutrophil extracellular trap formation. Chem. 2018c;4(2):334-58.

Jacobsen NR, Møller P, Clausen PA, Saber AT, Micheletti C, Jensen KA, Wallin H, Vogel U. Biodistribution of carbon nanotubes in animal models. Basic Clin Pharmacol Toxicol. 2017;121 Suppl 3:30-43.

Jones SW, Roberts RA, Robbins GR, Perry JL, Kai MP, Chen K, Bo T, Napier ME, Ting JP, Desimone JM, Bear JE. Nanoparticle clearance is governed by Th1/Th2 immunity and strain background. J Clin Invest. 2013;123(7):3061-73.

Johnson MM, Mendoza R, Raghavendra AJ, Podila R, Brown JM. Contribution of engineered nanomaterials physicochemical properties to mast cell degranulation. Sci Rep. 2017;7:43570.

Kagan VE, Konduru NV, Feng W, Allen BL, Conroy J, Volkov Y, Vlasova II, Belikova NA, Yanamala N, Kapralov A, Tyurina YY, Shi J, Kisin ER, Murray AR, Franks J, Stolz D, Gou P, Klein-Seetharaman J, Fadeel B, Star A, Shvedova AA. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. Nat Nanotechnol. 2010;5(5):354-9.

Katwa P, Wang X, Urankar RN, Podila R, Hilderbrand SC, Fick RB, Rao AM, Ke PC, Wingard CJ, Brown JM. A carbon nanotube toxicity paradigm driven by mast cells and the IL-$_{33}$/ST$_2$ axis. Small. 2012;8(18):2904-12.

Kurapati R, Mukherjee SP, Martín C, Bepete G, Vázquez E, Pénicaud A, Fadeel B, Bianco A. Degradation of single-layer and few-layer graphene by neutrophil myeloperoxidase. Angew Chem Int Ed Engl. 2018;57(36):11722-11727.

Larsen ST, Jackson P, Poulsen SS, Levin M, Jensen KA, Wallin H, Nielsen GD, Koponen IK. Airway irritation, inflammation, and toxicity in mice following inhalation of metal oxide nanoparticles. Nanotoxicology. 2016;10(9):1254-62.

Li R, Ji Z, Chang CH, Dunphy DR, Cai X, Meng H, Zhang H, Sun B, Wang X, Dong J, Lin S, Wang M, Liao YP, Brinker CJ, Nel A, Xia T. Surface interactions with compartmentalized cellular phosphates explain rare earth oxide nanoparticle hazard and provide opportunities for safer design. ACS Nano. 2014;8(2):1771-83.

Li R, Ji Z, Qin H, Kang X, Sun B, Wang M, Chang CH, Wang X, Zhang H, Zou H, Nel AE, Xia T. Interference in autophagosome fusion by rare earth nanoparticles disrupts autophagic flux and regulation of an interleukin-1β producing inflammasome. ACS Nano. 2014;8(10):10280-92.

Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-35.

Medzhitov R. Inflammation 2010: new adventures of an old flame, Cell, 140 (2010) 771-776.

Morimoto Y, Hirohashi M, Ogami A, Oyabu T, Myojo T, Todoroki M, Yamamoto M, Hashiba M, Mizuguchi Y, Lee BW, Kuroda E, Shimada M, Wang WN, Yamamoto K, Fujita K, Endoh S, Uchida K, Kobayashi N, Mizuno K, Inada M, Tao H, Nakazato T, Nakanishi J, Tanaka I. Pulmonary toxicity of well-dispersed multi-wall carbon nanotubes following inhalation and intratracheal instillation. Nanotoxicology. 2012;6(6):587-99.

Mortimer GM, Butcher NJ, Musumeci AW, Deng ZJ, Martin DJ, Minchin RF. Cryptic epitopes of albumin determine mononuclear phagocyte system clearance of nanomaterials. ACS Nano. 2014;8(4):3357-66.

Muñoz LE, Bilyy R, Biermann MH, Kienhöfer D, Maueröder C, Hahn J, Brauner JM, Weidner D, Chen J, Scharin-Mehlmann M, Janko C, Friedrich RP, Mielenz D, Dumych T, Lootsik MD, Schauer C, Schett G, Hoffmann M, Zhao Y, Herrmann M. Nanoparticles size-dependently initiate self-limiting NETosis-driven inflammation. Proc Natl Acad Sci U S A. 2016;113(40):E5856-E5865.

Nygaard UC, Samuelsen M, Marioara CD, Løvik M. Carbon nanofibers have IgE adjuvant capacity but are less potent than nanotubes in promoting allergic airway responses. Biomed Res Int. 2013;2013:476010.

Noël C, Simard JC, Girard D. Gold nanoparticles induce apoptosis, endoplasmic reticulum stress events and cleavage of cytoskeletal proteins in human neutrophils. Toxicol In Vitro. 2016;31:12-22.

Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018;18(2):134-147.

Poulsen SS, Jackson P, Kling K, Knudsen KB, Skaug V, Kyjovska ZO, Thomsen BL, Clausen PA, Atluri R, Berthing T, Bengtson S, Wolff H, Jensen KA, Wallin H, Vogel U. Multi-walled carbon nanotube physicochemical properties predict pulmonary inflammation and genotoxicity. Nanotoxicology. 2016;10(9):1263-75.

Ramadi KB, Mohamed YA, Al-Sbiei A, Almarzooqi S, Bashir G, Al Dhanhani A, Sarawathiamma D, Qadri S, Yasin J, Nemmar A, Fernandez-Cabezudo MJ, Haik Y, Al-Ramadi BK. Acute systemic exposure to silver-based nanoparticles induces hepatotoxicity and NLRP3-dependent inflammation. Nanotoxicology. 2016;10(8):1061-74.

Russier J, Ménard-Moyon C, Venturelli E, Gravel E, Marcolongo G, Meneghetti M, Doris E, Bianco A. Oxidative biodegradation of single- and multi-walled carbon nanotubes. Nanoscale. 2011;3(3):893-6.

Rydman EM, Ilves M, Koivisto AJ, Kinaret PA, Fortino V, Savinko TS, Lehto MT, Pulkkinen V, Vippola M, Hämeri KJ, Matikainen S, Wolff H, Savolainen KM, Greco D, Alenius H. Inhalation of rod-like carbon nanotubes causes unconventional allergic airway inflammation. Part Fibre Toxicol. 2014;11:48.

Sayan M, Mossman BT. The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. Part Fibre Toxicol. 2016;13(1):51.

Shvedova AA, Kapralov AA, Feng WH, Kisin ER, Murray AR, Mercer RR, St Croix CM, Lang MA, Watkins SC, Konduru NV, Allen BL, Conroy J, Kotchey GP, Mohamed BM, Meade AD, Volkov Y, Star A, Fadeel B, Kagan VE. Impaired clearance and enhanced pulmonary inflammatory/fibrotic response to carbon nanotubes in myeloperoxidase-deficient mice. PLoS One. 2012;7(3):e30923.

Scoville DK, Botta D, Galdanes K, Schmuck SC, White CC, Stapleton PL, Bammler TK, MacDonald JW, Altemeier WA, Hernandez M, Kleeberger SR, Chen LC, Gordon T, Kavanagh TJ. Genetic determinants of susceptibility to silver nanoparticle-induced acute lung inflammation in mice. FASEB J.;31(10):4600-4611.

Tsugita M, Morimoto N, Tashiro M, Kinoshita K, Nakayama M. SR-B1 Is a silica receptor that mediates canonical inflammasome activation. Cell Rep. 2017;18(5):1298-1311.

Vogt C, Pernemalm M, Kohonen P, Laurent S, Hultenby K, Vahter M, Lehtiö J, Toprak MS, Fadeel B. Proteomics analysis reveals distinct corona composition on magnetic nanoparticles with different surface coatings: implications for interactions with primary human macrophages. PLoS One. 2015;10(10):e0129008.

Yu Q, Wang H, Peng Q, Li Y, Liu Z, Li M. Different toxicity of anatase and rutile TiO(2) nanoparticles on macrophages: Involvement of difference in affinity to proteins and phospholipids. J Hazard Mater. 2017;335:125-134.

4.6. Neurotoxicity of Nanomaterials

Both incidental and intentional exposure (nanomedicine) to nanomaterials can have deleterious effects on the central nervous system (CNS). ENMs can, in principle, reach the brain from the systemic circulation by passing the brain-blood brain barrier, or by direct translocation *via* the olfactory bulb following inhalation (as reported in rodents). Crossing of the blood-brain barrier can occur through endothelial tight junctions for particles smaller than 6 nm or through transcytosis for larger particles (Cupaioli et al., 2014). For example, Ag nanoparticles were shown to penetrate the meningeal barrier *in vitro* using excised porcine meninges mounted on Franz diffusion cells (Mauro et al., 2016). In addition, particles can potentially gain access to the brain following the disruption of the blood brain barrier permeability. Such alterations have been reported *in vitro* for TiO_2 nanoparticles as well as for silver nanoparticles in conjunction with an inflammatory response and modified expression of tight junction proteins (Brun et al., 2012, Xu et al., 2015). Similarly, *in vivo* studies reported an increase in the permeability of the blood-brain barrier following exposure (intravenous, intraperitoneal, intracarotid, intracerebrovascular) of rodents to silver, copper and aluminium nanoparticles (Sharma et al., 2009). The effects were later shown to be size-dependent with smaller particles being more neurotoxic (blood-brain barrier breakdown, edema, cellular damage), especially for the very young and old rodents (Sharma et al., 2013). In addition, neurotoxic effects were more pronounced for silver and copper nanoparticles as compared with aluminium nanoparticles (Sharma et al., 2013). Secondly, studies have shown that particles can translocate from the nose to the brain *via* the olfactory bulb (Oberdörster et al., 2004). Ag nanoparticles and ultrafine carbon particles were shown to translocate to the brain *via* the olfactory bulb and sensory nerve endings in the respiratory tract, respectively, after inhalational exposure in rats (Patchin et al., 2016) Oberdörster et al., 2004). In addition, MnO nanoparticles were reported to translocate to the brain of rats through a similar route (Elder et al., 2006). A more recent study identified magnetite nanoparticles which were believed to originate from olfactory bulb transport in human brain samples, and the authors postulated a connection with Alzheimer's disease (Maher et al., 2016). However, it was not conclusively proven that the particles originated from an external source (air pollution) as opposed to being of biogenic origin (Gieré, 2016). Human studies on the effects of ENMs are scarce, nonetheless, environmental exposure to air pollution particles has previously been associated with CNS inflammation and markers of neurodegeneration in highly polluted areas in Mexico (Calderon-Garciduenas et al., 2008a, *idem*, 2008b). However, it is unclear to which

extent these effects are related to the ultrafine (< 100 nm) particulate fraction or to other organic components adsorbed at the surface of the particles.

Carbon-based nanomaterials

Carbon nanotubes (CNTs) have been envisioned as drug delivery agents to target the CNS (Guo et al., 2017). In terms of toxicity, multi-walled CNTs were reported to be neurotoxic and to reduce differentiation of PC12 cells, due to the high content of residual iron in the CNTs (Meng et al., 2013). In addition, multi-walled CNTs were shown to inhibit hippocampal CA1 glutamatergic synaptic transmission in rat hippocampal slices by whole-cell patch clamp evaluation (Chen et al., 2014) and reduce the axonal regeneration in neurons collected from the dorsal root ganglia (Wu et al., 2012). Animal studies reported behavioural changes more evident for multi-walled CNTs as compared with single-walled CNTs following intraperitoneal exposure (Gholamine et al., 2017). In addition, intra-peritoneal exposure to multi-walled CNTs induced autophagy, altered expression of NR2B (a subunit of NMDA receptor) and synaptophysin in hippocampus as well as impaired cognitive function in rats (Gao et al., 2015).

Metal nanoparticles

Silver nanoparticles were reported to induce neurotoxicity in human embryonic stem cell (hESC)-derived glutamatergic neurons (Begum et al., 2016). The effect was dose- and coating-dependent and was related to alteration of neurite outgrowth, increased production of ROS, modulation of Ca^{2+} influxes and changes in phosphorylation of GSK-3β and Tau proteins consistent with patterns observed in Alzheimer's disease (Begum et al. 2016). In addition, silver nanoparticles were shown to form f-actin inclusions, interfere with cytoskeletal organization and neurite extension in rat SVZ neural stem cells (Cooper and Spitzer, 2015). On the other hand, the toxicity of silver nanoparticles towards astrocytes seems to be less pronounced; in response to silver nanoparticles, astrocytes upregulated metallothioneins which may contribute to a protective effect on other brain cells (Hohnholt et al., 2013). Similarly, microglial cells were shown to reduce toxicity of silver nanoparticles towards dopaminergic neurons by upregulating the hydrogen sulphide (H_2S)-synthesizing enzyme cystathionine-γ-lyase that mediates the formation of non-reactive Ag_2S (Gonzalez-Carter et al., 2017). Serra et al. (2018) developed a novel tool for the contextualization of ENM effects in relation to human diseases,

and suggested, on the basis of similarities between the transcriptional profiles with other chemicals and drugs that certain metal and metal oxide nanoparticles are linked to neurodegenerative diseases such as amyotrophic lateral sclerosis. Further studies are needed to validate such predictions.

Several animal studies have shown that silver nanoparticles are distributed into the brain of rats following oral exposure (Kim et al., 2008, van der Zande et al., 2012) and accumulated into the olfactory bulb following inhalation exposure in rats (Patchin et al., 2016). The latter study also revealed activation of microglial cells (Patchin et al., 2016). Other *in vivo* studies reported that silver nanoparticles altered the expression of Bcl-2/Bax and induced apoptosis in the hippocampus following intraperitoneal exposure in rats (Ghooshchian et al., 2017). Moreover, silver nanoparticles interfered with the formation of myelin sheaths and altered the expression of myelin proteins albeit without behavioural changes following oral exposure in rats (Dąbrowska-Bouta et al., 2016). These studies raise concern regarding the unregulated uses of colloidal silver as 'health' products. It is important to note that there is no dietary requirement for silver, and, therefore, no such thing as a silver 'deficiency'.

Metal oxide nanoparticles

Cerium oxide nanoparticles (nanoceria) bear anti-oxidant properties that make them appealing for both industrial (fuel catalyst) and pharmacological purposes, for instance in the treatment of neurodegenerative diseases (Walkey et al., 2015). Nanoceria was shown to modulate the levels of brain-derived neurotrophic factor and increase neuronal survival in an *in vitro* 'model' of Alzheimer's disease (D'Angelo et al., 2009) and promoted neuronal differentiation and alter expression of genes involved in the antioxidant defense in PC12 cells (Ciofani et al., 2013, Ciofani et al., 2014). On the other hand, nanoceria was reported to inhibit neuronal differentiation (mouse neural stem cells and primary human embryonic stem cells) and interfered with the cytoskeletal organization and formation of the neuronal growth cone (Gliga et al., 2017).

Several *in vivo* studies have revealed beneficial effects of nanoceria in rat brain ischemic stroke model (Kim et al., 2012), mouse multiple sclerosis model (Heckman et al., 2013) and age-related macular degeneration (Kyosseva et al., 2013). It deserves to be noted that some of these disease

models display alterations of the blood-brain barrier which increases the bioavailability of nanoceria, as the bioavailability of nanoceria in the CNS would otherwise be marginal. On the other hand, other *in vivo* studies have shown potential neurotoxic effects following exposure to nanoceria. Hardas et al. (2012) reported that a single intravenous administration of nanoceria (5 nm) induced prooxidant effects in the brain 30 days post-exposure in the absence of brain translocation and that those changes are similar to the age- or Alzheimer's disease-related effects. In another study, the same group revealed that a single intravenous administration of nanoceria (30 nm) elicited a so-called hierarchical oxidative stress response in the rat hippocampus with a peak at day 30 and resolution at day 90 post-exposure (Hardas et al., 2014). The authors noted that the levels of nanoceria in the brain were very low and much of it could be attributed to nanoceria in the blood vessels perfusing the brain (Hardas et al., 2014).

Overall, in light of the known neurotoxicity of metals, there is a concern that metal-based nanoparticles, should they reach the brain, may exert adverse effects in humans (Karlsson et al., 2015). Further research is warranted, and epidemiological studies, eg., of welders who are exposed to nanoparticles (Andujar et al., 2014; Dierschke et al., 2017) may also shed light on the possible link between metal particles and neurotoxicity.

References

Andujar P, Simon-Deckers A, Galateau-Sallé F, Fayard B, Beaune G, Clin B, Billon-Galland MA, Durupthy O, Pairon JC, Doucet J, Boczkowski J, Lanone S. Role of metal oxide nanoparticles in histopathological changes observed in the lung of welders. Part Fibre Toxicol. 2014;11:23.

Begum, A. N., J. S. Aguilar, L. Elias and Y. Hong (2016). "Silver nanoparticles exhibit coating and dose-dependent neurotoxicity in glutamatergic neurons derived from human embryonic stem cells." NeuroToxicology 57(Supplement C): 45-53.

Brun, E., M. Carrière and A. Mabondzo (2012). "In vitro evidence of dysregulation of blood–brain barrier function after acute and repeated/long-term exposure to TiO2 nanoparticles." Biomaterials 33(3): 886-896.

Calderon-Garciduenas, L., A. Mora-Tiscareno, E. Ontiveros, G. Gomez-Garza, G. Barragan-Mejia, J. Broadway, S. Chapman, G. Valencia-Salazar, V. Jewells, R. R. Maronpot, C. Henriquez-Roldan, B. Perez-Guille, R. Torres-Jardon, L. Herrit, D. Brooks, N. Osnaya-Brizuela, M. E. Monroy, A. Gonzalez-Maciel, R. Reynoso-Robles, R. Villarreal-Calderon, A. C. Solt and R. W. Engle (2008a). "Air pollution, cognitive deficits and brain abnormalities: a pilot study with children and dogs." Brain Cogn 68(2): 117-127.

Calderon-Garciduenas, L., A. C. Solt, C. Henriquez-Roldan, R. Torres-Jardon, B. Nuse, L. Herritt, R. Villarreal-Calderon, N. Osnaya, I. Stone, R. Garcia, D. M.

Brooks, A. Gonzalez-Maciel, R. Reynoso-Robles, R. Delgado-Chavez and W. Reed (2008b). "Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults." Toxicol Pathol 36(2): 289-310.

Chen, T., J. Yang, H. Zhang, G. Ren, Z. Yang and T. Zhang (2014). "Multi-walled carbon nanotube inhibits CA1 glutamatergic synaptic transmission in rat's hippocampal slices." Toxicol Lett 229(3): 423-429.

Ciofani, G., G. G. Genchi, I. Liakos, V. Cappello, M. Gemmi, A. Athanassiou, B. Mazzolai and V. Mattoli (2013). "Effects of cerium oxide nanoparticles on PC12 neuronal-like cells: proliferation, differentiation, and dopamine secretion." Pharm Res 30(8): 2133-2145.

Ciofani, G., G. G. Genchi, B. Mazzolai and V. Mattoli (2014). "Transcriptional profile of genes involved in oxidative stress and antioxidant defense in PC12 cells following treatment with cerium oxide nanoparticles." Biochim Biophys Acta 1: 495-506.

Cooper, R. J. and N. Spitzer (2015). "Silver nanoparticles at sublethal concentrations disrupt cytoskeleton and neurite dynamics in cultured adult neural stem cells." Neurotoxicology 48: 231-238.

Cupaioli, F. A., F. A. Zucca, D. Boraschi and L. Zecca (2014). "Engineered nanoparticles. How brain friendly is this new guest?" Prog Neurobiol 119-120: 20-38.

D'Angelo, B., S. Santucci, E. Benedetti, S. Di Loreto, R. Phani, S. Falone, F. Amicarelli, M. P. Ceru and A. Cimini (2009). "Cerium oxide nanoparticles trigger neuronal survival in a human Alzheimer disease model by modulating BDNF pathway." Current Nanoscience 5(2): 167-176.

Dąbrowska-Bouta, B., M. Zięba, J. Orzelska-Górka, J. Skalska, G. Sulkowski, M. Frontczak-Baniewicz, S. Talarek, J. Listos and L. Strużyńska (2016). "Influence of a low dose of silver nanoparticles on cerebral myelin and behavior of adult rats." Toxicology 363-364(Supplement C): 29-36.

Dierschke K, Isaxon C, Andersson UBK, Assarsson E, Axmon A, Stockfelt L, Gudmundsson A, Jönsson BAG, Kåredal M, Löndahl J, Pagels J, Wierzbicka A, Bohgard M, Nielsen J. Acute respiratory effects and biomarkers of inflammation due to welding-derived nanoparticle aggregates. Int Arch Occup Environ Health. 2017;90(5):451-463.

Elder, A., R. Gelein, V. Silva, T. Feikert, L. Opanashuk, J. Carter, R. Potter, A. Maynard, Y. Ito, J. Finkelstein and G. Oberdorster (2006). "Translocation of inhaled ultrafine manganese oxide particles to the central nervous system." Environ Health Perspect 114(8): 1172-1178.

Gao, J., X. Zhang, M. Yu, G. Ren and Z. Yang (2015). "Cognitive deficits induced by multi-walled carbon nanotubes via the autophagic pathway." Toxicology 337(Supplement C): 21-29.

Gholamine, B., I. Karimi, A. Salimi, P. Mazdarani and L. A. Becker (2017). "Neurobehavioral toxicity of carbon nanotubes in mice:Focus on brain-derived neurotrophic factor messenger RNA and protein." Toxicology and Industrial Health 33(4): 340-350.

Ghooshchian, M., P. Khodarahmi and F. Tafvizi (2017). "Apoptosis-mediated neurotoxicity and altered gene expression induced by silver nanoparticles." Toxicol Ind Health 33(10): 757-764.

Gieré R. Magnetite in the human body: biogenic vs. anthropogenic. Proc Natl Acad Sci U S A. 2016;113(43):11986-11987.

Gliga, A. R., K. Edoff, F. Caputo, T. Kallman, H. Blom, H. L. Karlsson, L. Ghibelli, E. Traversa, S. Ceccatelli and B. Fadeel (2017). "Cerium oxide nanoparticles inhibit differentiation of neural stem cells." Sci Rep 7(1): 9284.

Gonzalez-Carter, D. A., B. F. Leo, P. Ruenraroengsak, S. Chen, A. E. Goode, I. G. Theodorou, K. F. Chung, R. Carzaniga, M. S. Shaffer, D. T. Dexter, M. P. Ryan and A. E. Porter (2017). "Silver nanoparticles reduce brain inflammation and related neurotoxicity through induction of H2S-synthesizing enzymes." Sci Rep 7: 42871.

Guo, Q., X. T. Shen, Y. Y. Li and S. Q. Xu (2017). "Carbon nanotubes-based drug delivery to cancer and brain." J Huazhong Univ Sci Technolog Med Sci 37(5): 635-641.

Hardas, S. S., R. Sultana, G. Warrier, M. Dan, R. L. Florence, P. Wu, E. A. Grulke, M. T. Tseng, J. M. Unrine, U. M. Graham, R. A. Yokel and D. A. Butterfield (2012). "Rat brain pro-oxidant effects of peripherally administered 5 nm ceria 30 days after exposure." Neurotoxicology 33(5): 1147-1155.

Hardas, S. S., R. Sultana, G. Warrier, M. Dan, P. Wu, E. A. Grulke, M. T. Tseng, J. M. Unrine, U. M. Graham, R. A. Yokel and D. A. Butterfield (2014). "Rat hippocampal responses up to 90 days after a single nanoceria dose extends a hierarchical oxidative stress model for nanoparticle toxicity." Nanotoxicology 1: 155-166.

Heckman, K. L., W. DeCoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone and J. S. Erlichman (2013). "Custom cerium oxide nanoparticles protect against a free radical mediated autoimmune degenerative disease in the brain." ACS Nano 7(12): 10582-10596.

Hohnholt, M. C., M. Geppert, E. M. Luther, C. Petters, F. Bulcke and R. Dringen (2013). "Handling of iron oxide and silver nanoparticles by astrocytes." Neurochem Res 38(2): 227-239.

Karlsson, H., Toprak, M. S., and Fadeel, B. (2015). Toxicity of Metal and Metal Oxide Nanoparticles. In: *Handbook on the Toxicology of Metals*. (G. F. Nordberg, B. A. Fowler, and M. Nordberg, Eds.), pp. 75-112. Academic Press.

Kim, C. K., T. Kim, I. Y. Choi, M. Soh, D. Kim, Y. J. Kim, H. Jang, H. S. Yang, J. Y. Kim, H. K. Park, S. P. Park, S. Park, T. Yu, B. W. Yoon, S. H. Lee and T. Hyeon (2012). "Ceria nanoparticles that can protect against ischemic stroke." Angew Chem Int Ed Engl 51(44): 11039-11043.

Kim, Y. S., J. S. Kim, H. S. Cho, D. S. Rha, J. M. Kim, J. D. Park, B. S. Choi, R. Lim, H. K. Chang, Y. H. Chung, I. H. Kwon, J. Jeong, B. S. Han and I. J. Yu (2008). "Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats." Inhal Toxicol 20(6): 575-583.

Kyosseva, S. V., L. Chen, S. Seal and J. F. McGinnis (2013). "Nanoceria inhibit expression of genes associated with inflammation and angiogenesis in the retina of Vldlr null mice." Exp Eye Res 116: 63-74.

Maher, B. A., I. A. M. Ahmed, V. Karloukovski, D. A. MacLaren, P. G. Foulds, D. Allsop, D. M. A. Mann, R. Torres-Jardón and L. Calderon-Garciduenas (2016). "Magnetite pollution nanoparticles in the human brain." Proc Natl Acad Sci U S A. 2016;113(39):10797-801.

Mauro, M., M. Crosera, M. Bovenzi, G. Adami and F. L. Filon (2016). "Pilot study on in vitro silver nanoparticles permeation through meningeal membrane." Colloids Surf B Biointerfaces 146: 245-249.

Meng, L., A. Jiang, R. Chen, C. Z. Li, L. Wang, Y. Qu, P. Wang, Y. Zhao and C. Chen (2013). "Inhibitory effects of multiwall carbon nanotubes with high iron impurity on viability and neuronal differentiation in cultured PC12 cells." Toxicology 313(1): 49-58.

Oberdörster, G., Z. Sharp, V. Atudorei, A. Elder, R. Gelein, W. Kreyling and C. Cox (2004). "Translocation of Inhaled Ultrafine Particles to the Brain." Inhalation Toxicology 16(6-7): 437-445.

Patchin, E. S., D. S. Anderson, R. M. Silva, D. L. Uyeminami, G. M. Scott, T. Guo, L. S. Van Winkle and K. E. Pinkerton (2016). "Size-Dependent Deposition, Translocation, and Microglial Activation of Inhaled Silver Nanoparticles in the Rodent Nose and Brain." Environ Health Perspect 6: 6.

Serra A, Letunic I, Fortino V, Handy RD, Fadeel B, Tagliaferri R, Greco D. insideNANO: a systems biology framework to contextualize the mechanism-of-action of engineered nanomaterials. Sci Rep. 2018 [in press].

Sharma, A., D. F. Muresanu, R. Patnaik and H. S. Sharma (2013). "Size- and age-dependent neurotoxicity of engineered metal nanoparticles in rats." Mol Neurobiol 48(2): 386-396.

Sharma, H. S., S. F. Ali, S. M. Hussain, J. J. Schlager and A. Sharma (2009). "Influence of engineered nanoparticles from metals on the blood-brain barrier permeability, cerebral blood flow, brain edema and neurotoxicity. An experimental study in the rat and mice using biochemical and morphological approaches." J Nanosci Nanotechnol 9(8): 5055-5072.

van der Zande, M., R. J. Vandebriel, E. Van Doren, E. Kramer, Z. Herrera Rivera, C. S. Serrano-Rojero, E. R. Gremmer, J. Mast, R. J. B. Peters, P. C. H. Hollman, P. J. M. Hendriksen, H. J. P. Marvin, A. A. C. M. Peijnenburg and H. Bouwmeester (2012). "Distribution, Elimination, and Toxicity of Silver Nanoparticles and Silver Ions in Rats after 28-Day Oral Exposure." ACS Nano 6(8): 7427-7442.

Walkey, C., S. Das, S. Seal, J. Erlichman, K. Heckman, L. Ghibelli, E. Traversa, J. F. McGinnis and W. T. Self (2015). "Catalytic Properties and Biomedical Applications of Cerium Oxide Nanoparticles." Environ Sci Nano 2(1): 33-53.

Wu, D., E. S. Pak, C. J. Wingard and A. K. Murashov (2012). "Multi-walled carbon nanotubes inhibit regenerative axon growth of dorsal root ganglia neurons of mice." Neuroscience Letters 507(1): 72-77.

Xu, L., M. Dan, A. Shao, X. Cheng, C. Zhang, R. A. Yokel, T. Takemura, N. Hanagata, M. Niwa and D. Watanabe (2015). "Silver nanoparticles induce tight junction disruption and astrocyte neurotoxicity in a rat blood-brain barrier primary triple coculture model." Int J Nanomedicine 10: 6105-6118.

4.7. Gastro-Intestinal Effects of Nanomaterials

Metal-based and polymeric nanoparticles have found increasing uses in the food packaging sectors leading to human exposure following ingestion of the nanomaterial-containing substances. Hence, metal oxide nanoparticles, such as titanium dioxide (TiO_2, E171), silicon dioxide (SiO_2, E551), and magnesium oxide (MgO, E530) are extensively used as food additives as whiteners, viscosity enhancers and fluxing agents and are authorized in food, consumer and medicinal products (Chaudhry et al., 2008; Bouwmeester et al., 2009; Europen Union, 2008a, 2008b; European Commission, 1994; US FDA, 2002). Furthermore, silver nanoparticles have been used as anti-bacterial agents in food packaging. It is estimated that an average person in a developed country consumes between 10^{12} and 10^{14} man-made fine (diameter 0.1–1 μm) to ultrafine (diameter <100 nm) particles every day specifically from food additives (Lomer et al., 2002). Secondary exposure to nanoparticles through the GI-tract can also occur as a consequence of respiratory exposures (Jepson et al., 2017). Once inside the GI-tract, nanoparticles can react with the surrounding secreted mucus and glycocalyx composed of biomolecules such as, pepsin, proteins, glycoproteins, carbohydrates, fats and lipids present in the GI tract lining through surface interactions forming a '*bio-corona'* (Behrens et al., 2002; Crater et al, 2010). It has been observed that anionic muco-adhesive nanoparticles diffuse 20-30 times faster than cationic nanoparticles and that the composition of the mucus affected their transportation (Crater et al, 2010). Interaction of nanoparticles with the glycocalyx is dependent upon the region of the GI tract, as nanoparticles pass more easily through the small intestine as compared to the large intestine due to thinner glycocalyx presenting less obstruction to the nanoparticles. Nanoparticles also face high acidic pH and ionic strength gastric juice in the digestive system. Therefore, depending upon the physicochemical properties of the individual nanoparticles, they may dissolve into ions, or aggregate/agglomerate into large particles before reaching the epithelial lining of the GI tract. Ag nanoparticles (60 nm) were found to agglomerate in the presence of gastric digestion proteins; however, the nanoparticles reverted into individual entities when intestinal digestion occurred indicating that they reached the epithelial lining of the GI tract as individual nanoparticles (Walczak et al., 2013). Similarly, Mwilu et al. (2013) investigated the interactions between synthetic gastric fluid and silver nanoparticles of different sizes, with and without coating agents and observed changes in the physico-chemical composition of the nanoparticles within 15 min of contact along with the release of Ag ions leading to the formation of a precipitation of silver chloride on their surface.

While there is a knowledge gap in terms of the actual site of uptake of nanoparticles in the GI tract and the underlying mechanism of translocation, it is presumed that the physicochemical properties play a major role and the nanoparticles are taken up by the epithelial lining cells through clatherin- and caveoli-dependent endocytosis (Hansen et al., 2009). Other uptake mechanism that might exist are 'presorption' and passage through the tight junctions of the epithelial lining of the GI tract under special circumstances (Jepson et al., 2017). ENMs can also accumulate at Peyer's patches with a large concentration of M cells, and/or further translocate to the lymphocytes present in the Peyer's patch across the intestinal epithelium barrier (Lomer et al., 2002; Kernéis et al., 1997).

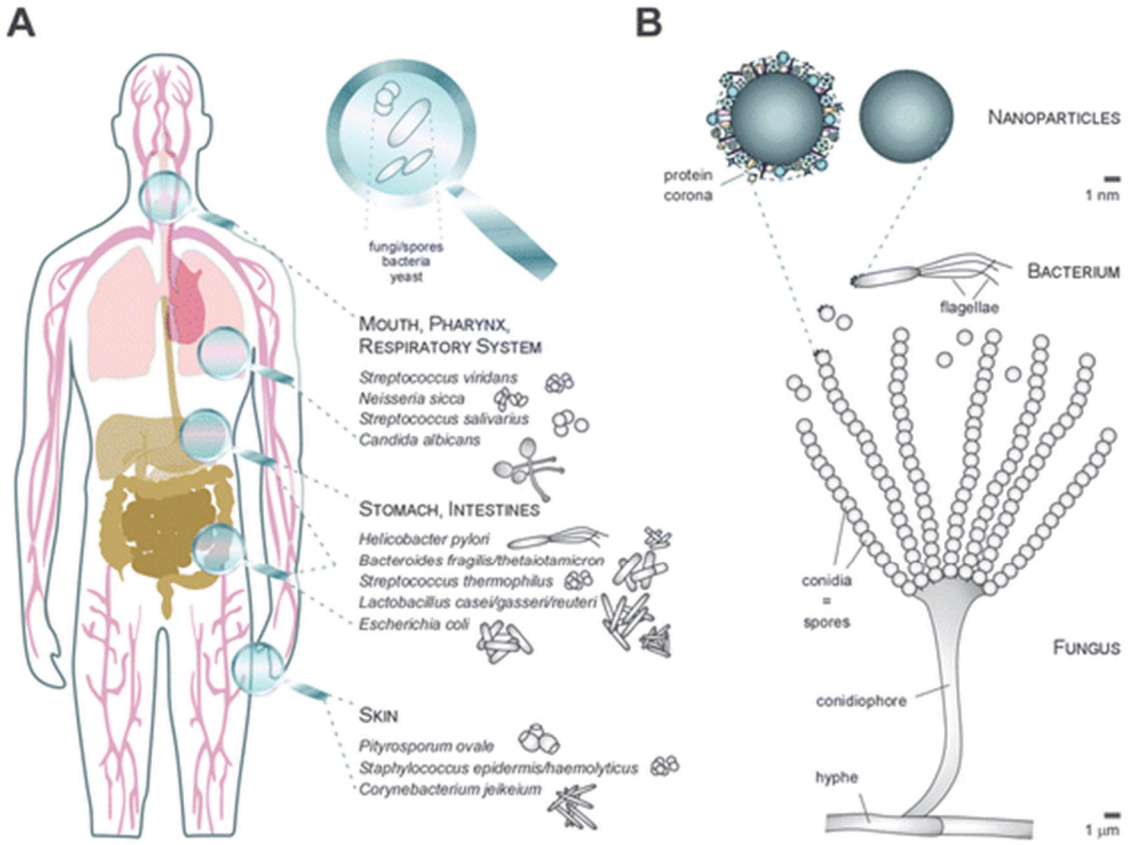


Figure 12. The human microbiome in contact with nanomaterials. (A) Microorganisms are present at all major exposure sites. (B) The bio-corona may influence the interaction of nanomaterials with microorganisms, such as bacteria or fungal spores. From: Docter et al. 2015.

Engulfment of nanomaterials by the immune cells may induce inflammatory reactions in the GI tract, as well as, further translocation of non-biodegradable nanomaterials to secondary organs such as liver, spleen and kidneys through the circulatory system causing secondary organ toxicity. Several *in vitro* models of the epithelial barrier have been

developed for the study of the interaction of ENMs with the GI tract, ranging from monolayers of a single cell type (often Caco-2 colorectal adenocarcinoma cells) to more complex co-cultures incorporating M cells and mucus-secreting cells. Co_3O_4 nanoparticles have been found to be non-cytotoxic in Caco-2 cells with low uptake rate (Mwilu et al., 2017), whilem Ag nanoparticles have been found to induce toxicity in Caco-2 cells (Lichtenstein et al., 2015; Böhmert et al., 2014). Kucki et al. (2017) reported that the uptake of graphene oxide is dependent on the differentiation status of Caco-2 cells. Differentiated Caco-2 cells present with a dense apical brush border of close-packed microvilli, and tight junctions. Ingestion of nanomaterials may interfere with the normal metabolic activity of the GI tract such as, reduction in the dietary absorption of iron and promotion in the development of Crohn's disease (Lomer et al., 2002; Mahler et al., 2012). ENMs may also interfere with the natural microbiome of the gut directly or indirectly by transforming the GI tract's natural environment and intestinal microbiota homeostasis with induction of 'dysbiosis'. The gut microbiota is thought to contain vast numbers of microorganisms, including at least 1,000 different species of known bacteria, the vast majority of which belong to the phyla, *Firmicutes* and *Bacteroidetes* (Ley et al., 2008). The GI tract and the intestinal microbiota display a symbiotic relationship with the microbiota contributing through the extraction of energy from food, synthesis of vitamins and amino acids, and helping as a barrier against pathogens (Tappenden et al., 2007). In fact, microbiomes are positioned at all major sites of nanoparticle exposure, i.e., in the lungs, on the skin, and in the gut (Docter et al., 2015) (Figure 12). However, there are so far only a small handful of studies of ENMs and the gut microbiome. Rats fed silver nanoparticles of various sizes for 13 weeks showed a general increase in the levels of gram-negative bacteria in the ileum (Williams et al., 2015). The 10-nm particles led to a decrease in levels of *Firmicutes*, particularly for members of the genus *Lactobacillus* (William et al., 2015). PVP and citrate coated silver nanoparticles of varying sizes were found to not to influence the microbiome of the gut (Wilding et al., 2016), while van den Brule et al. (2016) reported microbial alterations in the gut of mice exposed to silver nanoparticles. The bacterial disturbances recorded were similar to those reported in metabolic and inflammatory diseases, such as obesity. Chen et al. (2018) reported that single-walled CNTs promoted intestinal injuriy in mice at the acute dose of 2.5 mg/kg per day, including increased histological lesion scores, intestinal permeability, and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) secretion. Analysis of gut microbiota composition using 16S rRNA gene sequencing revealed that the CNTs induced significant shifts of the predominant phyla from *Firmicutes* to *Bacteroidetes*, and an increased abundance of proinflammatory bacteria.

Li et al. (2018) examined the effects of anatase and rutile TiO_2 nanoparticles on gut microbiota “at doses equivalent to those consumed by people who love to eat candies”. The results showed that titanium accumulated in the spleen, lung, and kidney, but had no significant effects on organ histology. TiO_2 nanoparticles did not decrease gut microbiota diversity, but shifted the composition of the microbiota; the most influenced phylum was *Proteobacteria*, which was significantly increased by rutile but not by anatase TiO_2. Thus, the crystalline phase of particles may play a role in mediating the intestinal impact of TiO_2 nanoparticles (Li et al., 2018).

Concluding remarks

One of the primary reason for the paucity of data on GI tract interactions of ENMs is the absence of relevant study models. Commonly used *in vitro* and *in vivo* test systems have their limitations. Single cell types such as Caco-2 cells cannot represent the complexity of the GI tract while *in vivo* assays frequently suffer from a lack of correlation between observations in animal models and effects in humans due to intrinsic genetic differences (Mahto et al., 2015; Kuempel et al., 2006; McKim et al., 2010). Moreover, the doses of ENMs administered to animals are, in most cases, highly unrealistic. Finally, *in vivo* experiments are obviously encumbered by ethical issues. It is therefore important to invest in more realistic *in vitro* models such as microfluidic systems that may provide a representation of the GI tract and yield results closer to those observed under *in vivo* conditions.

References

Behrens I, Pena AI, Alonso MJ, Kissel T. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm Res. 2002; 19(8):1185-93.

Bouwmeester H, Dekkers S, Noordam MY, Hagens WI, Bulder AS, de Heer C, ten Voorde SE, Wijnhoven SW, Marvin HJ, Sips AJ. Review of health safety aspects of nanotechnologies in food production. Regul Toxicol Pharmacol. 2009; 53(1):52-62.

Böhmert L, Girod M, Hansen U, Maul R, Knappe P, Niemann B, Weidner SM, Thünemann AF, Lampen A. Analytically monitored digestion of silver nanoparticles and their toxicity on human intestinal cells. Nanotoxicology. 2014 Sep;8(6):631-42.

Chaudhry Q, Scotter M, Blackburn J, Ross B, Boxall A, Castle L, Aitken R, Watkins R. Applications and implications of nanotechnologies for the food sector. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2008; 25(3):241-58.

Chen H, Zhao R, Wang B, Zheng L, Ouyang H, Wang H, Zhou X, Zhang D, Chai Z, Zhao Y, Feng W. Acute oral administration of single-walled carbon nanotubes increases intestinal permeability and inflammatory responses: association with the changes in gut microbiota in mice. Adv Healthc Mater. 2018;7(13):e1701313.

Crater JS, Carrier RL. Barrier properties of gastrointestinal mucus to nanoparticle transport. Macromol Biosci. 2010; 10(12):1473-83.

Docter D, Westmeier D, Markiewicz M, Stolte S, Knauer SK, Stauber RH. The nanoparticle biomolecule corona: lessons learned - challenge accepted? Chem Soc Rev. 2015;44(17):6094-121.

EU Official Journal of the European Union, 2008a. Commission Directive 2008/ 84/ Ec of 27 August 2008 Laying Down Specific Purity Criteria on Food Additive Other Than Colors and Sweeteners.

EU Official Journal of the European Union, 2008b. Regulation (Ec) No. 1333/ 2008 of the European Parliament and of council of 16 December 2008 on Food Additives.

European Commission 1994. European Parliament and Council Directive 94/ 36/ EC of June 30 1994 on colors in foodstuffs. Off. J. Eur. Communities L237, 13-29.

Hansen GH, Rasmussen K, Niels-Christiansen LL, Danielsen EM. Endocytic trafficking from the small intestinal brush border probed with FM dye. Am J Physiol Gastrointest Liver Physiol. 2009 Oct;297(4):G708-15.

Jepson MA, Bouwmeester H. In Chapter: Gastrointestinal system. In: *Adverse effects of Engineered nanomaterials: Exposure, Toxicology, and Impact on Human Health* (Second Edition). Eds: Fadeel B, Pietroiusti A, Shvedova A. Academic Press, pp: 381 – 396.

Kernéis S, Bogdanova A, Kraehenbuhl JP, Pringault E. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. Science. 1997; 277(5328).

Kucki M, Diener L, Bohmer N, Hirsch C, Krug HF, Palermo V, Wick P. Uptake of label-free graphene oxide by Caco-2 cells is dependent on the cell differentiation status. J Nanobiotechnology. 2017;15(1):46.

Kuempel ED, Tran CL, Castranova V, Bailer AJ. Lung dosimetry and risk assessment of nanoparticles: evaluating and extending current models in rats and humans. Inhal Toxicol. 2006 Sep;18(10):717-24.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. Science. 2008 Jun 20;320(5883):1647-51.

Li J, Yang S, Lei R, Gu W, Qin Y, Ma S, Chen K, Chang Y, Bai X, Xia S, Wu C, Xing G. Oral administration of rutile and anatase TiO_2 nanoparticles shifts mouse gut microbiota structure. Nanoscale. 2018;10(16):7736-7745.

Lichtenstein D, Ebmeyer J, Knappe P, Juling S, Böhmert L, Selve S, Niemann B, Braeuning A, Thünemann AF, Lampen A. Impact of food components during in vitro digestion of silver nanoparticles on cellular uptake and cytotoxicity in intestinal cells. Biol Chem. 2015 Nov;396(11):1255-64.

Lomer MC, Thompson RP, Powell JJ. Fine and ultrafine particles of the diet: influence on the mucosal immune response and association with Crohn's disease. Proc Nutr Soc. 2002; 61(1):123-30.

Mahler GJ, Esch MB, Tako E, Southard TL, Archer SD, Glahn RP, Shuler ML. Oral exposure to polystyrene nanoparticles affects iron absorption. Nat Nanotechnol. 2012; 7(4):264-71.

Mahto SK, Charwat V, Ertl P, Rothen-Rutishauser B, Rhee SW, Sznitman J. Microfluidic platforms for advanced risk assessments of nanomaterials. Nanotoxicology. 2015; 9(3):381-95.

McKim JM Jr. Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance. Comb Chem High Throughput Screen. 2010; 13(2):188-206.

Mwilu, S. K., El Badawy, A. M., Bradham, K., Nelson, C., Thomas, D., Scheckel, K. G., et al. (2013). Changes in silver nanoparticles exposed to human synthetic stomach fluid: effects of particle size and surface chemistry. *Sci. Total Environ*. 447:90–98.

Schmid K, Riediker M. Use of nanoparticles in Swiss Industry: a targeted survey. Environ Sci Technol. 2008 Apr 1;42(7):2253-60.

Tappenden KA, Deutsch AS. The physiological relevance of the intestinal microbiota: Contributions to human health. Journal of the American College of Nutrition. 2007;26(6):679S–S83S.

U.S. Food and Drug Administration, 2002. Code of Federal Regulations Title 21-Food and Drugs 21 CFR 73.2575, listing of color additives exempted from certification.

van den Brule S, Ambroise J, Lecloux H, Levard C, Soulas R, De Temmerman PJ, Palmai-Pallag M, Marbaix E, Lison D. Dietary silver nanoparticles can disturb the gut microbiota in mice. Part Fibre Toxicol. 2016;13(1):38.

Walczak AP, Fokkink R, Peters R, Tromp P, Herrera Rivera ZE, Rietjens IM, Hendriksen PJ, Bouwmeester H. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology. 2013 Nov;7(7):1198-210.

Wilding LA, Bassis CM, Walacavage K, Hashway S, Leroueil PR, Morishita M, Maynard AD, Philbert MA, Bergin IL. Repeated dose (28-day) administration of silver nanoparticles of varied size and coating does not significantly alter the indigenous murine gut microbiome. Nanotoxicology. 2016;10(5):513-20.

Williams, K., Milner, J., Boudreau, M. D., Gokulan, K., Cerniglia, C. E., and Khare, S. (2015). Effects of subchronic exposure of silver nanoparticles on intestinal

microbiota and gut-associated immune responses in the ileum of Sprague-Dawley rats. *Nanotoxicology*. 9:279–289.

Wüstenberg, T. (ed) (2014) Nanocellulose, in: *Cellulose and Cellulose Derivatives in the Food Industry: Fundamentals and Applications*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

4.8. Genotoxicity and Carcinogenicity of Nanomaterials

Today, a number of particles or fumes containing (nano)particles are regarded as carcinogenic to humans following exposure *via* inhalation as assessed by IARC. These exposures include, for example, particulate matter in outdoor air pollution, crystalline silica dust, tobacco smoking, and welding fumes. For ENMs, there is still very limited information from human and animal studies regarding potential carcinogenicity. However, genotoxicity as evidenced by DNA damage (i.e., DNA strand breaks, oxidatively damaged DNA) as well as mutations has been studied in various models. Here, nanomaterial-induced genotoxicity is discussed with emphasis on mechanisms and on the suitability of common methods used to measure genotoxicity. Furthermore, an overview of genotoxicity and carcinogenicity of metal and carbon-based nanomaterials is provided.

Mechanisms and methods

The mechanisms of nanoparticle-induced genotoxicity are not completely understood. Furthermore, it is not clear if there are any 'nano-specific' effects (Donaldson and Poland, 2013). Genotoxicity can be classified as either "primary genotoxicity" or "secondary genotoxicity". The primary genotoxicity refers to genotoxicity from the nanoparticles themselves (direct or indirect) whereas secondary genotoxicity refers to the induction of genotoxicity *via* ROS generated during particle-elicited inflammation (Schins and Knaapen, 2007). Direct DNA interaction could represent a more nano-specific mechanism due to the fact that small nanoparticles may reach the nucleus through nuclear pore complexes (NPC) (Nabiev et al., 2007). Even though NPCs consist of a tube with a diameter of approximately 30 nm, also larger nanoparticles have been observed in the nucleus. One possibility is that these may gain access to the DNA in dividing cells when the nuclear membrane disassembles. In one of the few studies claiming a size-dependent interaction with DNA, gold nanoparticles with a distinct particle size of 1.4 nm were shown to interact in a unique manner with the major grooves of DNA, which could account for the toxicity of these small nanoparticles (Tsoli et al., 2005). However, most studies suggest that the main mechanisms are not direct interaction with DNA, but rather an indirect cause of damage due to, e.g., oxidative stress as a result of either oxidant-generating properties of the nanoparticles themselves or ROS formation due to interaction with mitochondria. Another possibility is that nanoparticles interact with the mitotic spindle apparatus, centrioles or their associated proteins and thereby cause aneugenic effecs, i.e., loss or gain of chromosomes in daughter cells. As discussed by Sargent et al. (2010), the long thin tubular-

shaped carbon nanotubes show a striking similarity to cellular microtubules, suggesting a potential to interact with the mitotic spindle as well as the motor proteins that separate the chromosomes during cell division.

The most commonly used assays for assessing genotoxicity of nanoparticles is the comet assay and analysis of micronuclei (MN) *in vitro* (Golbamaki et al., 2015; Magdolenova et al., 2014). The general understanding is that the assays can be used for the evaluation of ENMs, but with some caution. Regarding the comet assay, for example, photocatalytically active nanoparticles, such as some TiO_2, may cause false positives in the presence of light (Karlsson et al., 2015). A recent study showed a positive response in comet assay for gold nanoparticles, but the authors concluded that the response observed may be "false positive" due to additional DNA damage formed during the assay performance as a result of direct interaction between Au nanoparticles and nucleoid DNA (George et al., 2017). This has also been suggested in other studies (Karlsson et al., 2015) and such interactions may, indeed, result in additional DNA breaks. To what extent such damage formed during the assay performance cause false positives, rather than slightly exaggerated damage, remains to be elucidated. For analysis of micronuclei, the use of cytochalasin B (used to score MN specifically in once-divided binucleated cells) can inhibit uptake of the nanoparticles (KEMI, 2016). Furthermore, bacterial cells are sometimes used for mutagenicity testing in the Ames test, but these tests are not recommended for mutagenicity testing of ENMs.

Metal-based nanoparticles

Golbamaki et al. (2015) published a review focusing on genotoxicity studies of various metal oxides. According to this report, TiO_2 was the most studied material (63 studies) followed by ZnO (25 studies), SiO_2 (18 studies) and CuO (14 studies). Whereas basically all published studies on human cells exposed to CuO showed positive findings, the effects from the other metal oxides were more divergent (Golbamaki et al., 2015). In another recent report from the Swedish Chemicals Agency (KEMI, 2016), all published *in vivo* studies investigating genotoxicity of SiO_2, TiO_2, Au, Ag and CNTs were compiled. For all materials, both positive and negative studies were reported. No effects on blood or bone marrow cells were observed following administration of ENMs *via* the pulmonary route. In contrast, local effects in lung cells were observed convincingly for CNTs, but not for the other nanoparticles (though very few studies are available

for the other ENMs). Several studies investigating TiO_2 nanoparticles showed genotoxicity following oral exposure. Furthermore, both gold and silver nanoparticles were found to be genotoxic following injection. Based on the available literature it is apparent that the administration route is important when assessing genotoxicity of ENMs. Regarding silver nanoparticles, a recent summary of the results from 16 *in vivo* studies showed that genotoxicity was recorded in most cases (Fewtrell et al., 2017).

Although certain metal dusts such as NiO (not exclusively nanoparticles) are classified as carcinogenic to humans, there is presently limited data on chronic effects following exposure to inhaled metal-based manufactured nanoparticles. The metal oxide nanoparticle type that has been mostly studied is TiO_2. The carcinogenic potential of TiO_2 (bulk and nano) was reviewed and assessed by IARC (2010) and ECHA (2016). The overall evaluation by IARC is that TiO_2 is possibly carcinogenic to humans (Group 2B). The IARC assessment was based on four epidemiological studies as well as experimental carcinogenicity studies in rats, mice and hamsters by different routes of exposure (oral, inhalation, intratracheal, subcutaneous and intraperitoneal administrations). The human carcinogenicity data did not show an association between occupational exposure to TiO_2 and risk for cancer, although some methodological limitations were observed (IARC, 2010). Both inhalation and intratracheal instillation studies showed increased risk of lung cancers in rats. On the other hand, tumor incidence was not increased in intratracheally instilled hamsters or mice. Moreover, oral, subcutaneous and intraperitoneal administrations did not cause a significant increase in the frequency of any type of tumour in mice or rats. As discussed by ECHA, studies supporting a promoting potential of TiO_2 nanoparticles are now also available and the final proposed to classification is an inhalation specific classification (Cat 1B- H350i). TiO_2 was not proposed to be placed in category 2 since malignant tumors were reported in more than one experiment of adequate quality (ECHA, 2016).

“a recent summary of the results from 16 *in vivo* studies on silver nanoparticles showed that genotoxicity was recorded in most cases”

An important question relates to the possible difference in carcinogenic potency between nano- and micron-sized poorly-soluble particles. After compiling the current literature, a factor of 2.0–2.5 (referring to the dose

metrics mass concentration) was suggested (Gebel, 2012). It should be noted, however, that the human health relevance of lung tumors observed in rats after inhalation to very high doses of particles leading to particle "overload" conditions has been called into question (Valberg et al., 2009).

Carbon-based nanomaterials

IARC has assessed carcinogenicity of carbon black and concluded that the human epidemiological evidence was inconsistent since two of the three studies of carbon black production workers observed excess risk for lung cancer whereas other studies provided mixed evidence (IARC, 2010). On the other hand, three studies of female rats that inhaled carbon black and three additional studies of female rats exposed intratracheally found significant increases in the incidence of malignant lung tumors at high doses (overload conditions). High retained mass lung burdens and decreased lung clearance have been observed also in coal miners, which led IARC to conclude that animal cancer data obtained under conditions of impaired lung clearance are relevant to humans. The final assessment of carbon black by IARC is that it is possibly carcinogenic to humans (Group 2B) (IARC, 2010). IARC has also recently evaluated the carcinogenicity of carbon nanotubes and for this evaluation, a total of 11 cancer studies of various types of MWCNTs and SWCNTs in rats or mice were available. The group concluded that there was sufficient evidence of cancer in animals (two or more adequate studies) for one type of CNT (MWCNT-7), whereas there was limited evidence for other CNTs (Grosse et al., 2014). The positive studies were based on injections into the peritoneal or scrotal cavity and mesotheliomas. The overall IARC evaluation was thus that MWCNT-7, a long and rigid multi-walled CNT, was classified as possibly carcinogenic to humans (Group 2B) and SWCNTs and all other MWCNTs excluding MWCNT-7 were considered not classifiable as to their carcinogenicity to humans (Group 3). More recently, a 2-year study on rats demonstrated concentration dependent associations between inhalation of MWCNT-7 (0.02, 0.2 and 2 mg/m^3 for 6 h/day, 5 days/week) and development of lung carcinoma (mainly bronchiolo-alveolar carcinoma) (Kasai et al., 2016). However, no development of pleural mesothelioma was observed. Furthermore, a study showed tumor promoting effects in rats treated with methylcholantrene and MWCNT-7 (5 mg/m^3, 5 h/day, 5 days/week for 15 days) (Sargent et al., 2014). Bornholdt et al. (2017) profiled the genome-wide usage of transcription start sites and linked active enhancer regions in lungs of mice 24 h after intratracheal instillation of a single dose of MWCNT-7 and could show a massive gene regulatory response, where expression of key inflammatory genes was increased >100-fold 24 h after exposure to

MWCNTs. Chernova et al. (2017) recently reported that long, multi-walled CNTs replicated asbestos-induced mesothelioma following instillation (not inhalation) into the pleural cavity of mice. The authors presented evidence that hypermethylation of p16/Ink4a and p19/Arf in CNT and asbestos induced inflammatory lesions preceded mesothelioma, consistent with epigenetic alterations playing a role in cancer. Taken together, a picture is emerging that is consistent with the so-called pathogenic fibre paradigm insofar as long and rigid CNTs have been shown to be more pathogenic in rodents than the short or tangled (flexible) CNTs (Kuempel et al., 2017).

To summarize, many ENMs trigger genotoxicity *in vitro*, but the carcinogenic potential of ENMs remains largely unknown. Nevertheless, TiO_2, carbon black, and a specific variety of CNTs (so-called MWCNT-7) have been evalauted by IARC as being possibly carcinogenic to humans.

References

Bornholdt J, Saber AT, Lilje B, Boyd M, Jørgensen M, Chen Y, Vitezic M, Jacobsen NR, Poulsen SS, Berthing T, Bressendorff S, Vitting-Seerup K, Andersson R, Hougaard KS, Yauk CL, Halappanavar S, Wallin H, Vogel U, Sandelin A. Identification of gene transcription start sites and enhancers responding to pulmonary carbon nanotube exposure in vivo. ACS Nano. 2017;11(4):3597-3613.

Chernova T, Murphy FA, Galavotti S, Sun XM, Powley IR, Grosso S, Schinwald A, Zacarias-Cabeza J, Dudek KM, Dinsdale D, Le Quesne J, Bennett J, Nakas A, Greaves P, Poland CA, Donaldson K, Bushell M, Willis AE, MacFarlane M. Long-fiber carbon nanotubes replicate asbestos-induced mesothelioma with disruption of the tumor suppressor gene Cdkn2a (Ink4a/Arf). Curr Biol. 2017;27(21):3302-3314.e6.

Donaldson, K., and Poland, C. A. (2013). Nanotoxicity: challenging the myth of nano-specific toxicity. Current opinion in biotechnology 24(4), 724-34.

ECHA (2016). Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: Titanium Dioxide EC Number: 236-675-5; CAS Number 13463-67-7.

Fewtrell, L., Majuru, B., and Hunter, P. R. (2017). A re-assessment of the safety of silver in household water treatment: rapid systematic review of mammalian in vivo genotoxicity studies. Environmental health. 16(1), 66.

Gebel, T. (2012). Small difference in carcinogenic potency between GBP nanomaterials and GBP micromaterials. Archives of toxicology 86(7), 995-1007.

George, J. M., Magogotya, M., Vetten, M. A., Buys, A. V., and Gulumian, M. (2017). An investigation of the genotoxicity and interference of gold nanoparticles in commonly used in vitro mutagenicity and genotoxicity assays. Toxicological sciences. 156(1), 149-166.

Golbamaki, N., Rasulev, B., Cassano, A., Marchese Robinson, R. L., Benfenati, E., Leszczynski, J., and Cronin, M. T. (2015). Genotoxicity of metal oxide

nanomaterials: review of recent data and discussion of possible mechanisms. Nanoscale 7(6), 2154-98.
Grosse, Y., Loomis, D., Guyton, K. Z., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., et al. (2014). Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. The Lancet Oncology 15(13), 1427-8.
IARC (2010). Carbon Black, Titanium Dioxide, and Talc. IARC MONOGRAPHS VOLUME 93
Karlsson, H. L., Di Bucchianico, S., Collins, A. R., and Dusinska, M. (2015). Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity? Environmental and molecular mutagenesis 56(2), 82-96.
Kasai, T., Umeda, Y., Ohnishi, M., Mine, T., Kondo, H., Takeuchi, T., Matsumoto, M., and Fukushima, S. (2016). Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. Particle and fibre toxicology 13(1), 53.
KEMI (2016). Nanomaterials and genotoxicity – a literature review. 1-57.
Kuempel, E. D., Jaurand, M. C., Moller, P., Morimoto, Y., Kobayashi, N., Pinkerton, K. E., Sargent, L. M., Vermeulen, R. C., Fubini, B., and Kane, A. B. (2017). Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans. Critical reviews in toxicology 47(1), 1-58.
Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., and Dusinska, M. (2014). Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8(3), 233-78.
Nabiev, I., Mitchell, S., Davies, A., Williams, Y., Kelleher, D., Moore, R., Gun'ko, Y. K., Byrne, S., Rakovich, Y. P., Donegan, J. F., et al. (2007). Nonfunctionalized nanocrystals can exploit a cell's active transport machinery delivering them to specific nuclear and cytoplasmic compartments. Nano letters 7(11), 3452-61.
Sargent, L. M., Porter, D. W., Staska, L. M., Hubbs, A. F., Lowry, D. T., Battelli, L., Siegrist, K. J., Kashon, M. L., Mercer, R. R., Bauer, A. K., et al. (2014). Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. Particle and fibre toxicology 11, 3.
Sargent, L. M., Reynolds, S. H., and Castranova, V. (2010). Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects. Nanotoxicology 4, 396-408.
Schins, R. P., and Knaapen, A. M. (2007). Genotoxicity of poorly soluble particles. Inhalation toxicology 19 Suppl 1, 189-98.
Tsoli, M., Kuhn, H., Brandau, W., Esche, H., and Schmid, G. (2005). Cellular uptake and toxicity of Au55 clusters. Small (Weinheim an der Bergstrasse, Germany) 1(8-9), 841-4.
Valberg, P. A., Bruch, J., and McCunney, R. J. (2009). Are rat results from intratracheal instillation of 19 granular dusts a reliable basis for predicting cancer risk? Regulatory toxicology and pharmacology : RTP 54(1), 72-83.

5. Emerging Approaches

5.1. Advanced In Vitro and Ex Vivo Models

Traditional approaches for chemical safety assessment often depend on animal studies, but a new paradigm, relying instead on so-called alternative testing strategies (ATS), based on *in vitro* assays and *in silico* predictive tools for testing chemicals, is is now emerging (Nel and Malloy, 2017). In the following sections, we discuss some of the emerging approaches in nanosafety research including advanced *in vitro* models, high-throughput screening (HTS), and systems biology/toxicology approaches, with examples of research conducted at IMM and elsewhere.

In vivo models

Animal testing is common in toxicology and rodents (rats and mice) are the most commonly used organism as a model of human diseases. Researchers use rodents because of their genetic, biological and behavioral similarities with human. Furthermore, rodents are small, easily housed, maintained and breed fast (Vandamme 2014, Parasuraman 2011). However, it is clear that the use of animals has its limitations: mice and humans take up substances differently, and metabolize them differently (Hartung, 2009). The sensitivity to toxicants significantly varies in rodent and human lungs and this is explained, in part, by the much higher expression of metabolizing enzymes (mostly belonging to the CYP superfamily) in rodent lungs compared to human lungs (Fröhlich et al., 2014). Many studies have shown that inhaled nanomaterials are retained in the body of animals for longer periods than human lungs. For instance, 75% of 100 nm carbon-based nanomaterials was retained for more than 48 h in hamster lungs (Geiser et al., 2010). Clearly, there is a need for an overhaul of (regulatory) toxicology (Hartung, 2009; Nel and Malloy, 2017).

In vitro models

A majority of studies related to toxic effects of nanoparticles performed to date have utilized cell cultures. Common (transformed) cell lines representing different organs include lung cells (e.g., human A549), liver cells (e.g., human HepG2), gastrointestinal cells (e.g., human Caco-2), skin (e.g., human HaCaT) and monocytes/macrophages (human THP-1, murine RAW264.7). In most studies, cells are exposed to particles suspended in a liquid (e.g., mixed into the cell medium). This will lead to interactions between the cell culture medium and the nanoparticles and to agglomeration of the nanoparticles, which could affect the biological response. The fact that nanoparticles can settle, diffuse and aggregate in

various ways depending on their size, density and surface chemistry, as well as the properties of the culture medium can indeed also affect the particle dose reaching the cells at the bottom of the culture dish. The behavior of nanoparticles in cell culture media could be a major explanation for reported differences in nanoparticle toxicity (Teeguarden et al., 2007) and questions related to dosimetry have been much highlighted during recent years. The cellular dose can be measured quantitatively using, e.g., inductively coupled plasma mass spectrometry (ICP-MS). An alternative approach is to estimate the delivered dose by modelling, for instance, using the "in vitro sedimentation, diffusion and dosimetry" (ISDD) model (Hinderliter et al., 2010; Thomas et al., 2018). One critical aspect highlighted in this work is the effective density and diameter of nanoparticle agglomerates in suspension. Indeed, for buoyant nanoparticles (polypropylene), the agglomerates will also have a lower density, and their suspended forms may not settle, but would instead float above the cells making it difficult to determine the dose-response relationship (Watson et al., 2016). Furthermore, cellular uptake of nanoparticles may differ depending on the cell type and even though most cells are phagocytic, professional phagocytes such as macrophages are much more proficient at engulfing nanoparticles, leading to a greater intracellular dose. Other factors such as the presence of serum and the formation of a so-called bio-corona also impact on *in vitro* toxicity of ENMs.

Air-liquid interface exposure

An alternative approach is to use direct exposure of (lung) cells at the air-liquid interface (ALI). In this case, the cells are cultured on transwell membranes with no cell culture medium covering the cells, thus enabling cell exposure to an aerosol of particles. Such exposure is more comparable to inhalation of nanoparticles. A variety of ALI cell exposure systems have been described in the literature (Aufderheide and Mohr, 1999; Lenz et al., 2009; Elihn et al, 2013). Most of them rely on diffusion and/or gravitational settling for deposition and since nanoparticles tend to follow the air stream, obtaining appropriate deposition onto the cells can be challenging (Elihn et al., 2013). One option to increase the deposition onto cells is to use electrostatic deposition, where the particles are first charged and deposition enhanced by an electrostatic force generated beneath the exposure chamber. Most of the procedures described in the literature have been developed in-house, but some systems are commercially available. These more realistic exposure scenarios may be a way forward and could enable better *in vitro-in vivo* correlations. However, it is presently unclear whether ALI systems show different

effects compared to conventional submerged cultures and whether these systems are better in predicting the impact of ENMs in exposed humans.

Most *in vitro* studies include one single exposure to ENMs. However, there are a few examples of repeated exposures of bronchial epithelial cells cultured at ALI (Leclercq et al., 2014, Chortarea et al., 2015, Boublil et al., 2013). Repeated MWCNT exposures of lung cell cultured at ALI elicited a limited biological impact over a three day period (Chortarea et al., 2015).

Long term in vitro studies

While the large majority of studies in nanotoxicology have focused on acute effects, there is a current demand for more chronic exposure scenarios (Johnston et al., 2013) using low doses that more closely resemble human exposure. Short-term studies can be useful for toxicity screening and prioritization purposes, but they are unable to provide comprehensive information on complex processes, e.g., the carcinogenic potential of ENMs. When designing *in vitro* long-term exposure studies it is important that the selected cell model can be maintained for long periods of time in culture while maintaining a stable phenotype. Several studies have been published in recent years using human bronchial epithelial cells (BEAS-2B) and human keratinocytes (HaCaT) for long-term exposures (Comfort el al., 2014, Wang et al., 2011, Luanpitpong et al., 2014). Wang et al. (2011) evaluated the tumorigenic potential of single-walled CNTs following low dose (0.02 μg/cm^2) exposure of BEAS-2B up to 24 weeks. The results indicated that long-term exposure to SWCNTs induced a tumorigenic phenotype (anchorage-independent cell growth, cell invasion, cell migration and p53-mediated apoptosis resistance) in BEAS-2B cells and the transformed cells formed tumors when injected into immunodeficient mice (Wang et al., 2011). In a follow-up study, the same authors provided evidence that long-term exposure to SWCNTs induced the induction of cancer stem cells (Luanpitpong et al., 2014). In addition, several long-term studies on metal nanoparticles have revealed a tumorigenic potential for titanium dioxide (Vales et al., 2015), cobalt nanoparticles (Annangi et al., 2015), and silver nanoparticles (Choo et al, 2016). Another study addressed the chronic effect of long-term (3 months), low-dose exposure

“several studies have reported the potential for oncogenic transformation in lung cells cultured for long periods of time in the presence of low doses of ENMs”

of human HaCaT cells to silver nanoparticles (50 nm) and reported induction of sustained cellular stress (activation of p38, increased Ki67 expression, and altered expression of stress related genes) (Comfort *et al.*, 2014). Using RNA-sequencing, Gliga et al. (2018) could show that exposure of BEAS-2B cells to a low dose (1 µg/mL) of silver nanoparticles (10 nm) for 6 weeks resulted in epithelial-to-mesenchymal transition and this was confirmed by using functional assays which showed an increased invasion index, anchorage independent cell growth, and cadherin switching.

Taken together, several studies have reported the potential for oncogenic transformation in lung cells cultured for long periods of time in the presence of low doses of ENMs. This is something that should be investigated further. Clearly, high-dose, acute toxicity tests will not suffice.

Advanced in vitro lung cell models

Physiologically relevant *in vivo*-like *in vitro* lung-mucosa models with primary cells cultured at air–liquid interface (ALI) are emerging as a realistic and efficient alternative for pulmonary toxicity testing and cell-cell interaction studies (Boublil et al., 2013, Pillai et al., 2014, Ji et al., 2017). Since lung tissue contains more than 40 different types of cells, mimicking the microenvironment *in vivo* requires *in vitro* systems involving specialized cells arranged in a realistic architecture (Rothen-Rutishauser et al., 2008). Pulmonary fibroblasts maintain structural integrity and tissue homeostasis by producing the extracellular matrix (ECM) and growth factors. Moreover, these cells are active participants in inflammatory responses via the local release of cytokines (Marshall et al., 2015). Other cells of interest that can be incorporated include macrophages and neutrophils, which are recruited to the lung in response to particle exposure (Rothen-Rutishauser et al., 2005). Multicellular models of bronchial mucosa have also been established (Ji et al., 2017, Bitterle et al., 2016, Scheffler et al., 2015, Mathis et al., 2013) and such models are also commercially available. Models composed of human fibroblasts together with human primary bronchial epithelial cells obtained from either healthy subjects or individuals with respiratory diseases such as chronic bronchitis have been established at IMM. Culturing at the air-liquid interface allows the epithelial cells to differentiate into ciliated cells, mucus producing cells and basal cells (Ji et al., 2017). The normal bronchial epithelium consists of 50-70% ciliated cells, up to 30% basal cells, up to 25% goblet cells, and 11% clara cells (now known as club cells). These models demonstrated an existing mucociliary defence, where particles

were trapped in the mucus layer and pulled together by the beating cilia which indicated a good *in vivo* resemblance (Ji et al., 2017). Such models, incorporating multiple cell types, allow cell- to cell interactions and cross-talk to be examined. In addition, models of normal mucosa and chronic bronchitis-like mucosa as well as models with cells from different patients enable comparison of responses between different patients groups. However, less research is done with alveolar mucosa models and to our knowledge there are no existing commercial alveolar mucosa models available. The alveolar mucosa consists of alveolar type I and type II cells. Alveolar type I (ATI) cells cover around 90% of the alveolar surface while the smaller alveolar type II (ATII) cells cover approximately 10% of the surface. The type I cells provide a large area for gas exchange and are involved in ion and protein transport. Several groups have developed methods to isolate rat and human ATII cells in primary culture (Mao et al., 2015). However, under most conditions primary ATII tended to differentiate into ATI cells and it is difficult to maintain surfactant-producing human ATII cells in culture (Bove et al.,2014). Most of the current alveolar models in the literature are based on tumorigenic or immortalized cell lines which lack biological relevance due to their genetic alterations and lack of expression of essential physiological functions like tight junctions (Lehmann et al., 2011, Ren et al., 2016, Tomasek et al., 2016). However, there are some cell lines that still maintain physiological functions. For instance, NCI-H441 cells exhibit high expression of junctional proteins ZO-1 and E-cadherin, seal-forming claudin-3, -4, -5 and Na^+-K^+-ATPase (Ren et al., 2016), making this a better option than other cell lines such as A549.

Organ-on-a-chip model systems

Traditional 2D culture models are widespread yet these models often fail in their prediction of *in vivo* outcomes. More advanced model systems, including 3D spheroid cultures (Pampaloni et al., 2007) and so-called organ-on-a-chip models may potentially provide a more realistic model for drug testing as well as for the toxicity testing of other chemicals and ENMs. The organ-on-a-chip is a microfluidic cell culture device created with microchip manufacturing methods that includes continuously perfused chambers in which cells are arranged to simulate tissue and organ level physiology (Bhatia and Ingber, 2014). Ingber and co-workers developed a ‘breathing’ organ-on-a-chip device that reconstitutes the functional alveolar-capillary interface of the human lung (Huh et al., 2010). Using this lung mimic, they could show that cyclic mechanical strain accentuates the toxic and inflammatory responses to silica nanoparticles. Mechanical strain also enhanced epithelial and endothelial uptake of nanoparticles and stimulated their transport into the underlying microvascular channel

(Huh et al., 2010). Furthermore, a human gut-on-a-chip device was constructed to analyze contributions of the microbiome to intestinal pathophysiology and dissect mechanisms in a manner that would not be possible using conventional *in vitro* systems (Kim et al., 2016). Oleaga et al. (2016) devised an *in vitro* model composed of cells representative of four organs under continuous flow conditions in serum-free medium. The authors could show functional activity of the cardiac, muscle, neuronal, and liver modules, and the pharmacological relevance of the integrated system was evaluated for 5 drugs with known side effects; the results of the drug treatments were in general agreement with published toxicity results from human and animal data. Overall, while such advanced models offer the very promising prospect of studying human physiology *in vitro* in a controlled manner, the movement towards microfluidic applications is still in its infancy, and such 'organ' systems need to be properly validated.

References

Annangi, B., Bach, J., Vales, G., Rubio, L., Marcos, R. & Hernandez, A. (2015). Long-term exposures to low doses of cobalt nanoparticles induce cell transformation enhanced by oxidative damage. *Nanotoxicology,* 9, 138-47.

Aufderheide, M., U. Mohr (2004). A modified CULTEX system for the direct exposure of bacteria to inhalable substances. *Exp Toxicol Pathol* 55(6), 451-454.

Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol. 2014;32(8):760-72.

Bitterle, E., Karg E., Schroeppel, A., Kreyling, W.G., Tippe, A., Ferron, G.A., Schmid, O (2006). Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere* 65, 1784-1790.

Boublil, L., Assemat, E., Borot, M.C., Boland, S., Martinon, L., Sciare, J., Baeza Squiban, A (2013). Development of a repeated exposure protocol of human bronchial epithelium in vitro to study the long-term effects of atmospheric particles. *Toxicol in vitro* 27, 533-542.

Bove, P.F., Dang, H., Cheluvaraju, C., Jones, L.C., Liu X., O´Neal, W.K. et al (2014). Breaking the in vitro alveolar type II cell proliferation barrier while retaining ion transport properties. *Am J Respir Cell Mol Biol* 50, 767-776.

Choo, W.H., Park, C.H., Jung, S.E., Moon, B., Ahn, H., Ryu, J.S., Kim, K.S., Lee, Y.H., Yu, I.J. & Oh, S.M. (2016). Long-term exposures to low doses of silver nanoparticles enhanced in vitro malignant cell transformation in non-tumorigenic BEAS-2B cells. *Toxicol In Vitro* 37, 41-49.

Comfort, K. K., Braydich-Stolle, L. K., Maurer E. I. and Hussain S. M. (2014). Less is more: long-term in vitro exposure to low levels of silver nanoparticles provides new insights for nanomaterial evaluation. *ACS Nano* 8(4), 3260-3271.

Chortarea, S., Clift, M.J.D., Vanhecke, D., Endes, C., Wick, P., Petri-Fink, A., Rothen-Rutishauser, B (2015). Repeated exposure to carbon nanotube-based

aerosols does not affect the functional properties of a 3D human epithelial airway model. *Nanotoxicology* 9, 983–993.

Derk, R., Davidson, D.C., Manke, A., Stueckle, T.A., Rojanasakul, Y., Wang, L.(2015). Potential in vitro model for testing the effect of exposure to nanoparticles on the lung alveolar epithelial barrier. *Sensing and Bio-Sensing Research* 3, 38–45.

Elihn, K., Cronholm, P., Karlsson H.L., Midander, K., Odnevall Wallinder, I., Möller L. (2013) Cellular dose of partly soluble Cu particle aerosols at the air-liquid interface using an in vitro lung cell exposure system. *J Aerosol Med Pulm Drug Deliv* 26, 84-93.

Fröhlich, E. and Salar-Behzadi, S (2014). Toxicological Assessment of Inhaled Nanoparticles: Role of in Vivo, ex Vivo, in Vitro, and in Silico Studies. *Int. J. Mol. Sci.* 15, 4795-4822.

Geiser, M. and Kreyling, W.G (2010). Deposition and biokinetics of inhaled nanoparticles. *Part. Fibre Toxicol.* 7, 2.

Gliga AR, Di Bucchianico S, Lindvall J, Fadeel B, Karlsson HL. RNA-sequencing reveals long-term effects of silver nanoparticles on human lung cells. Sci Rep. 2018;8(1):6668.

Hartung T. Toxicology for the twenty-first century. Nature. 2009;460(7252):208-12.

Hinderliter, P.M., K.R. Minard, G. Orr, W.B. Chrisler, B.D. Thrall, J.G. Pounds, J.G. Teeguarden (2010). ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. *Part Fibre Toxicol* 7:36.

Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. Science. 2010;328(5986):1662-8.

Ji, J., Hedelin, A., Malmlöf, M., Kessler, V., Seisenbaeva, G., Gerde, P., Palmberg, L (2017). Development of combining of human bronchial mucosa models with XposeALI® for exposure of air pollution nanoparticles. *PLoSOne* 12: e0170428.

Johnston, H., G. Pojana, S. Zuin, N. R. Jacobsen, P. Moller, S. Loft, M. Semmler-Behnke, C. McGuiness, D. Balharry, A. Marcomini, H. Wallin, W. Kreyling, K. Donaldson, L. Tran and V. Stone (2013). Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges. *Critical Reviews in Toxicology* 43(1), 1-20.

Kim HJ, Li H, Collins JJ, Ingber DE. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. Proc Natl Acad Sci U S A. 2016;113(1):E7-15.

Leclercq, B., Happillon, M., Antherieu, S., Hardy, E.M. Alleman, L.Y. , Grova, N., Perdrix, E. , et al (2016). Differential responses of healthy and chronic obstructive pulmonary diseased human bronchial epithelial cells repeatedly exposed to air pollution-derived PM4. *Environmental Pollution*, 218, 1074e1088.

Lehmann, A.D., Daum, N., Bur, M., Lehr, C.M., Gehr, .P, Rothen-Rutishauser, B,M (2011). An *in vitro* triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier. *Eur J Pharm Biopharm* 77, 398-406.

Lenz, A.G., T. Stoeger T, D. Cei, M. Schmidmeir, N. Semren, G. Burgstaller, et al (2014). Efficient bioactive delivery of aerosolized drugs to human pulmonary epithelial cells cultured in air-liquid interface conditions. *American journal of respiratory cell and molecular biology* 51(4), 526-35.

Luanpitpong S., Wang L., Castranova and V., Rojanasakul Y., Induction of stem-like cells with malignant properties by chronic exposure of human lung epithelial cells to single-walled carbon nanotubes. *Part Fibre Toxicol*, 2014, 11, 22.

Mathis, C., Poussin, C., Weisensee, D., Gebel, S., Hengstermann, A., Sewer, A., Belcastro, V., Xiang, Y., et al (2013). Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers. *Am J Physiol Lung Cell Mol Physiol* 304, 489–503.

Mukherjee, D., Porter, A., Mary, R., Schwander, S., Chung, K.F., Tetley, T., Zhang J, et al (2015). Modeling In Vivo Interactions of Engineered Nanoparticles in the Pulmonary Alveolar Lining Fluid. *Nanomaterials* 5, 1223-1249.

Nel AE, Malloy TF. Policy reforms to update chemical safety testing. Science. 2017;355(6329):1016-1018.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W. et al (2005). Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Particle and Fibre Toxicology* 2:8.

Osier, M., Öberdorster, G. (1997) Intratracheal inhalation vs intratracheal instillation: differences in particle effects. *Fundam. Appl. Toxicol*. 40, 220–227.

Oleaga C, Bernabini C, Smith AS, Srinivasan B, Jackson M, McLamb W, Platt V, Bridges R, Cai Y, Santhanam N, Berry B, Najjar S, Akanda N, Guo X, Martin C, Ekman G, Esch MB, Langer J, Ouedraogo G, Cotovio J, Breton L, Shuler ML, Hickman JJ. Multi-Organ toxicity demonstration in a functional human in vitro system composed of four organs. Sci Rep. 2016;6:20030.

Pillai, D.K., Sankoorikal, B.J.V., Johnson, E., Seneviratne, A.N., Zurko, J., Brown, K.J., Hathout, Y., Rose, M.C., 2014. Directional secretomes reflect polarity-specific functions in an in vitro model of human bronchial epithelium (2014). *Am. J. Respir. Cell. Mol. Biol.* 50, 292-300.

Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol. 2007;8(10):839-45.

Parasuraman, S (2011). Toxicological screening. J Pharmacol Pharmacother. 2, 74–79.

Ren, H., Birch, N.P., Suresh, V (2016). An optimized human cell culture model for alveolar epithelial transport. *PLoSONE* 11:e0165225.

Rothen-Rutishauser, B., F. Blank, C. Muhlfeld, P Gehr (2008). In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter. *Expert opinion on drug metabolism & toxicology* 4(8), 1075-89.

Rothen-Rutishauser, B.M., S.G. Kiama, P. Gehr (2005). A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *Am J Resp Cell Mol Biol* 32(4), 281-9.

Scheffler, S., Dieken, H., Krischenowski, O., Forster, C., Branscheid, D., Aufderheide, M (2015). Evaluation of E-cigarette liquid vapor and mainstream cigarette

smoke after direct exposure of primary human bronchial epithelial cells. *Int J Environ Res Public Health.* 12, 3915–25.

Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., et al (2008). Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: Inflammation, fibrosis, oxidative stress, and mutagenesis. *Am. J. Physiol. Lung Cell Mol.* Physiol. 295, 552–565.

Vales, G., Rubio, L. & Marcos, R., 2015. Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells. *Nanotoxicology,* 9, 568-78.

Vandamme, T.F (2014). Use of rodents as models of human diseases. J Pharm Bioallied Sci. 6(1), 2–9.

Wang., L, Luanpitpong, S., Castranova, V., Tse, W., Y., Lu, V., Pongrakhananon Y and Rojanasakul, Y (2011). Carbon nanotubes induce malignant transformation and tumorigenesis of human lung epithelial cells . *Nano Lett*, 2011, 11, 2796-2803.

Watson CY, DeLoid GM, Pal A, Demokritou P. Buoyant nanoparticles: implications for nano-biointeractions in cellular studies. Small. 2016;12(23):3172-3180.

Teeguarden, J.G., P.M. Hinderliter, G. Orr, B.D. Thrall, J.G. Pounds (2007). Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. *Toxicol Sci* 95(2), 300-12.

Thomas DG, Smith JN, Thrall BD, Baer DR, Jolley H, Munusamy P, Kodali V, Demokritou P, Cohen J, Teeguarden JG. ISD3: a particokinetic model for predicting the combined effects of particle sedimentation, diffusion and dissolution on cellular dosimetry for in vitro systems. Part Fibre Toxicol. 2018;15(1):6.

Tomasek, I., et al. (2016). Combined exposure of diesel exhaust particles and respirable Soufrière Hills volcanic ash causes a (pro-) inflammatory response in an in vitro multicellular epithelial tissue barrier model. *Particle and fibre toxicology* 13:67.

5.2. High-Throughput Screening (HTS)

High-throughput screening (HTS), originating from drug discovery (Macarron et al., 2011), provides an opportunity for large-scale testing of ENMs. HTS technology is a well-documented and proven instrument-driven scientific tool kit for expanding towards data-driven *in vitro* based safety assessment (Krewski et al., 2014). Several different types of HTS methods have been developed and adapted for ENMs during the last few years, including diverse methods for label-free cellular screening of nanomaterial uptake, cytotoxicity and genotoxicity, as well as high-content immunochemical analysis and high-throughput flow cytometry applicable to various biomarkers for, e.g., immunotoxicity, oxidative stress or fibrogenesis (Feliu and Fadeel, 2010; Damoiseaux et al., 2011; Nel et al., 2013; Collins et al., 2017). In addition, omics approaches including RNA sequencing are emerging and are expected to be useful in combination with other HTS approaches to reveal complex physiological and toxicological effects (Andersen et al., 2015; Grimm et al., 2016; Collins et al., 2017).

Methodological developments for HTS have involved the implementation of rigorous quality assurance methods for standard usage protocols, including liquid handling and performance monitoring. Problems of systematic bias and false discovery results are dealt with using data normalization and correction techniques under general data preprocessing protocols that include plate-specific and assay-specific error detection tests (Mpindi et al., 2015). The common generation of multiple replicate measurements under precise robotics-mediated handling steps ensures that the quality of HTS data is very high, and often better controlled than data generated by lower throughput methods, making the approach highly applicable to accelerated testing of ENMs in diverse conditions and provides a basis for identification of sources of variability, such as artifacts related to concentration, storage, exposure time, cell density, culture with or without serum or other supplements, and different dispersion and dilution protocols (reviewed in: Collins et al., 2017).

HTS facilitates comprehensive tiered testing strategies including toxicity assessment and high-content immunochemical assays followed by, or aligned with, omics analysis, leading to gradually broader characterization of intoxicating concentrations of selected, potentially class-representative nanomaterials (Thomas et al., 2013, Krewski et al., 2014, Stone et al., 2014, Grafström et al., 2015, Godwin et al., 2015, Collins et al., 2017). An

example of such a tiered strategy directly applicable for modernized "*in vitro* only-based" toxicological testing of ENMs under the 21st century toxicology (aka Tox21) paradigm is provided in Grafström et al. (2015). Thus, a tiered approach to ENM safety evaluation proceeds from physicochemical characterization (stage 1) to cell-based HTS (stage 2 and 3) and further to omics analyses, typically transcriptomics, of ENMs selected for more in-depth investigation (stage 4). Finally, high-risk ENMs are subject to the last step involving alternative animal models to validate the predictive models developed from the data in the previous steps (stage 5). Non-testing, i.e., structural and exposure-driven methods can be used if appropriate read-across hypotheses can be established in accordance with the Nano-Read Across Assessment Framework (RAAF) (ECHA, 2017). In stage 2, toxicity tests are typically applied for relative toxic potency ranking of multiple agents over a wide range of concentrations. Stage 3 broadens the number of toxicity endpoints of multiple agents in the narrowed concentration intervals considered relevant based on the stage 2 results (Grafström et al., 2015). Automated microscopy-based morphological assessments in stages 2 and 3 provide a high content dimension to such analyses, and also provide a secondary toxicity readout that controls for potential assay interference by the tested agents. In stage 4, class-representative agents with high intrinsic potency for exerting toxic effects are typically subjected to even broader characterization, including limited concentrations and time points based on the stage 2 and 3 results (Grafström et al., 2015). Typically, gene expression profiling then provides an opportunity to determine toxicology-relevant bio-identities of the tested agents representing "the selected few" group. In summary, tiered strategies allow for initial, relative toxicity ranking, substance prioritization, dose-response and point of departure calculations useful for quantitative *in vitro* to *in vivo* extrapolations, as well as the exploration or verification of novel or known pathways of toxicity, mode-of-action mechanisms, and, in a broader sense, adverse outcome pathways or AOPs (Grafström et al., 2015; Langley et al., 2015; Kohonen et al., 2014; Wetmore et al., 2015).

To define safe doses of tested substances, *in vitro*-only based risk assessment usually applies benchmark dose (BMD) modeling, which is also applicable to genes and particularly to gene sets that coincide broadly with the most sensitive toxicity endpoints (Thomas et al., 2013; Grafström et al., 2015). Furthermore, testing regimes can be expanded to a wide range of cell types and assays, and ENMs can also be tested for low-dose effects. Eventually, alternative animal models can be used to validate predictions and to assess translocation and toxicokinetic properties of high-risk ENMs. Lin et al. (2011) provided an example of automated

screening of metal oxides using zebrafish embryos. In most cases, HTS is performed using well-characterized and easily handled human monocultures, based on cancer tissue-derived or normal tissue-originating immortalized cell lines. However, the shift away from animal testing has brought more complex human models into focus, and such models are increasingly being applied and standardized to the HTS format (Pamies et al., 2017). Such models, inlucing stem-cell derived human cells and 3D spheroid cultures, have the potential to inform organ-specific toxic responses and biomarkers, and may also allow for toxicokinetics considerations (Alepee et al., 2014). On the other hand, depending on the aim of the study, less sophisticated, yet robust cell models are also relevant especially with regards to standardization, as long as quality can be assured and Good Cell Culture Practice is implemented (Pamies et al., 2017).

Data integration across structural, high-throughput, high-content, as well as pathway-based cellular assays, and omics profiling (and animal testing, if needed) gives the final picture on the nanomaterial activity. In order to evaluate toxicity in the context of existing knowledge, databases with each data type need to be utilized in conjunction with profiling efforts (Kohonen et al., 2014). Toxicity assessment can be seen both as a data-driven and a concept-driven activity. Connectivity mapping with gene expression or HTS data to match patterns from an unknown compound to an agent with known toxicity or mode-of-action is an example of a data-driven activity (Feliu et al., 2014, Grafström et al., 2015), while the identification of MIE and KE that lead to an adverse outcome is a concept-driven activity that facilitates evaluation of evidence for toxicity (refer to the chapter on AOPs). In parallel to the ongoing data accumulation, it is important to also develop well-standardized and documented bioinformatics workflows for toxicity assessment, especially for integration of various omics and HTS data (refer to the chapter on data management). Several EU projects have generated useful data on ENMs by using HTS approaches. In the EU-funded NANOMILE project, 14 ENMs comprised of CeO_2, Ag, TiO_2, ZnO and SiO_2 with different coatings and surface characteristics were tested at varying concentrations using high-throughput/high-content screening methods (Hansjosten et al., 2018). Cell lines derived from relevant organs such as liver, lung, GI-tract, and the immune system were utilized, and the authors found that differentiated liver cells appeared to be the most sensitive to ENMs. Overall, it became clear that adverse effects were dependent on the assay (endpoint) and cell line. Whereas Ag and ZnO nanoparticles were toxic in all cell types tested, SiO_2 nanoparticles, dependent on surface functionalization, appeared selectively toxic to

macrophages. Moreover, TiO_2 nanparticles affected liver and colon cells with induction of apoptosis. Some nanoparticles, namely CeO_2 did not score in any assays, although previous studies of the same material had shown inflammation and cytotoxicity after inhalation in a rat model (Hansjosten et al., 2018). Such discrepancies could perhaps be resolved by performing exposures under more realistic conditions, eg., at ALI (see above).

References

Alépée N, et al. State-of-the-art of 3D cultures (organs-on-a-chip) in safety testing and pathophysiology. ALTEX. 2014;31(4):441-77.

Andersen, M.E., McMullen, P.D., Krewski, D. Developing tools for defining and establishing pathways of toxicity. Arch Toxicol. 89, 809-12 (2015).

Collins A.R. et al. High throughput toxicity screening and intracellular detection of nanomaterials. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 9(1) (2017).

Damoiseaux R, George S, Li M, Pokhrel S, Ji Z, France B, Xia T, Suarez E, Rallo R, Mädler L, Cohen Y, Hoek EM, Nel A. No time to lose--high throughput screening to assess nanomaterial safety. Nanoscale. 2011;3(4):1345-60.

ECHA Read-Across Assessment Framework (RAAF). European Chemicals Agency; Helsinki, Finland. (2017).

Feliu N, Fadeel B. Nanotoxicology: no small matter. Nanoscale. 2010;2(12):2514-20.

Feliu N. et al. Next-generation sequencing reveals low-dose effects of cationic dendrimers in primary human bronchial epithelial cells. ACS Nano. 9(1):146-63 (2015).

Grafström, R., et al., Toward the replacement of animal experiments through the bioinformatics-driven analysis of 'omics' data from human cell cultures. Altern Lab Anim, 2015. 43(5): p. 325-32.

Grimm F.A. et al. A chemical-biological similarity-based grouping of complex substances as a prototype approach for evaluating chemical alternatives. Green Chem. 18(16):4407-4419 (2016).

Godwin H. et al. Nanomaterial categorization for assessing risk potential to facilitate regulatory decision-making. ACS Nano. 29(4):3409-17 (2015).

Hansjosten I, et al. Microscopy-based high-throughput assays enable multi-parametric analysis to assess adverse effects of nanomaterials in various cell lines. Arch Toxicol. 2018;92(2):633-649.

Kohonen P, Ceder R, Smit I, Hongisto V, Myatt G, Hardy B, Spjuth O, Grafström R. Cancer biology, toxicology and alternative methods development go hand-in-hand. Basic Clin Pharmacol Toxicol. 2014;115(1):50-8.

Krewski, D. et al. A framework for the next generation of risk science. Environmental Health Perspectives 122, 796-805 (2014).

Langley, G. et al. Lessons from toxicology: developing a 21st-century paradigm for medical research. Environ Health Perspect. 123, A268-72 (2015).

Lin SJ, Zhao Y, Xia T, Meng H, Ji ZX, et al. (2011) High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. ACS Nano 5: 7284-7295.

Macarron R, Banks MN, Bojanic D, Burns DJ, Cirovic DA, Garyantes T, Green DV, Hertzberg RP, Janzen WP, Paslay JW, Schopfer U, Sittampalam GS. Impact of high-throughput screening in biomedical research. Nat Rev Drug Discov. 2011;10(3):188-95.

Mpindi JP, Swapnil P, Dmitrii B, Jani S, Saeed K, Wennerberg K, Aittokallio T, Östling P, Kallioniemi O. Impact of normalization methods on high-throughput screening data with high hit rates and drug testing with dose-response data. Bioinformatics. 2015 Dec 1;31(23):3815-21.

Nel A, Xia T, Meng H, Wang X, Lin S, Ji Z, Zhang H. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc Chem Res. 2013;46(3):607-21.

Pamies D, Hartung T. 21st century cell culture for 21st century toxicology. Chem Res Toxicol. 2017;30(1):43-52.

Stone V. et al. ITS-NANO--prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Part Fibre Toxicol. 11:9 (2014).

Thomas, R.S. et al. Incorporating new technologies into toxicity testing and risk assessment: moving from 21st century vision to a data-driven framework. Toxicol Sci. 136, 4-18 (2013).

Wetmore BA. Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. Toxicology. 2015 Jun 5;332:94-101.

5.3. Systems Toxicology/Omics Approaches

Classical toxicological testing paradigms rely heavily on animal testing, despite ethical pressures to shift to *in vitro* alternatives. The 21st century toxicology paradigm (National Research Council, 2007; Collins et al., 2008) calls for a shift away from descriptive toxicology, based to a large extent on animal testing of toxicants one by one and with a multitude of functionally disconnected assays, towards a *predictive toxicology* grounded in a more solid understanding of the relevant toxicity and adverse outcomes in humans. A *systems toxicology* approach promises to shed new light on the interactions of ENMs with biological systems to reveal the causal connections between changes in the expression of genes, proteins or metabolites and the biological pathways that underlie the toxicity phenotypes (Sturla et al., 2014). Systems toxicology approaches rely on: (1) collection of large sets of experimental high-quality data by omics technologies; (2) utilization of bioinformatics and systems biology tools to identify hazard associated biomarkers and molecular networks to be used for predictive toxicology; and (3) validation of the predictive models by comparing numerical simulations with the actual experimental data. Key challenges are how to link reliably identified gene profiles/networks to toxicological phenotypes (immunotoxicity, genotoxicity, etc), how to demonstrate reliability and prediction accuracy of computational models in real life, and how to develop mechanism- or pathway-based testing strategies toward risk assessment (Hartung et al., 2017).

Systems toxicology analysis requires that omics data is generated using suitable models which permit the reliable measurement of end-points of relevant functional biological processes. Cell-based models are widely used, affordable and easy to standardize, but they lack the natural complexity of living organisms and are therefore not sufficient to meet all the demands in a systems toxicology analysis. Although toxicant-induced network perturbations can be identified in cell models it is difficult to confirm that the observed changes are predictive of true adverse reactions in humans or in environmental species. In order to model closely the complexity of organism physiology use of *in vivo* models is still needed to investigate several toxicological aspects. Recent advances have led to organ-on-a-chip models that incorporate some of the functional and anatomical properties of organ tissues and are therefore potentially more suitable to explore effects of complex cellular mechanisms (Bhatia and Ingber, 2014). Moreover, substantial effort has been made to validate *in vitro* derived hazard-associated features and pathways against state-of-the-art *in vivo* models. Quality and robustness of the omics data is a

prerequisite for any reliable systems toxicology analysis. Omics data quality control and proper pre-processing are crucial steps in the analysis process. Normalization methods and strategies for batch-effect corrections must be carefully selected based on the omics method and the platform used. Moreover, intra-laboratory and inter-laboratory reproducibility and intra-laboratory repeatability of the data generation must be carefully taken into account, to avoid 'noise', i.e., the generation of vast amounts of meaningless data (Fadeel, 2015). To increase reproducibility of the samples used for omics analysis it is suggested that biomolecules for omics analysis are purified centrally in a one laboratory in each project. If this is not possible then the use of SOPs for sample preparation is highly suggested. Similarly, omics data generation should be centralized and carefully planned to avoid introduction of undesired batch effects. Furthermore, data analysis methods, correction of p-values for multiple testing and cut-off methods for positivity should be agreed jointly among all investigators to avoid differences in the analysis strategy.

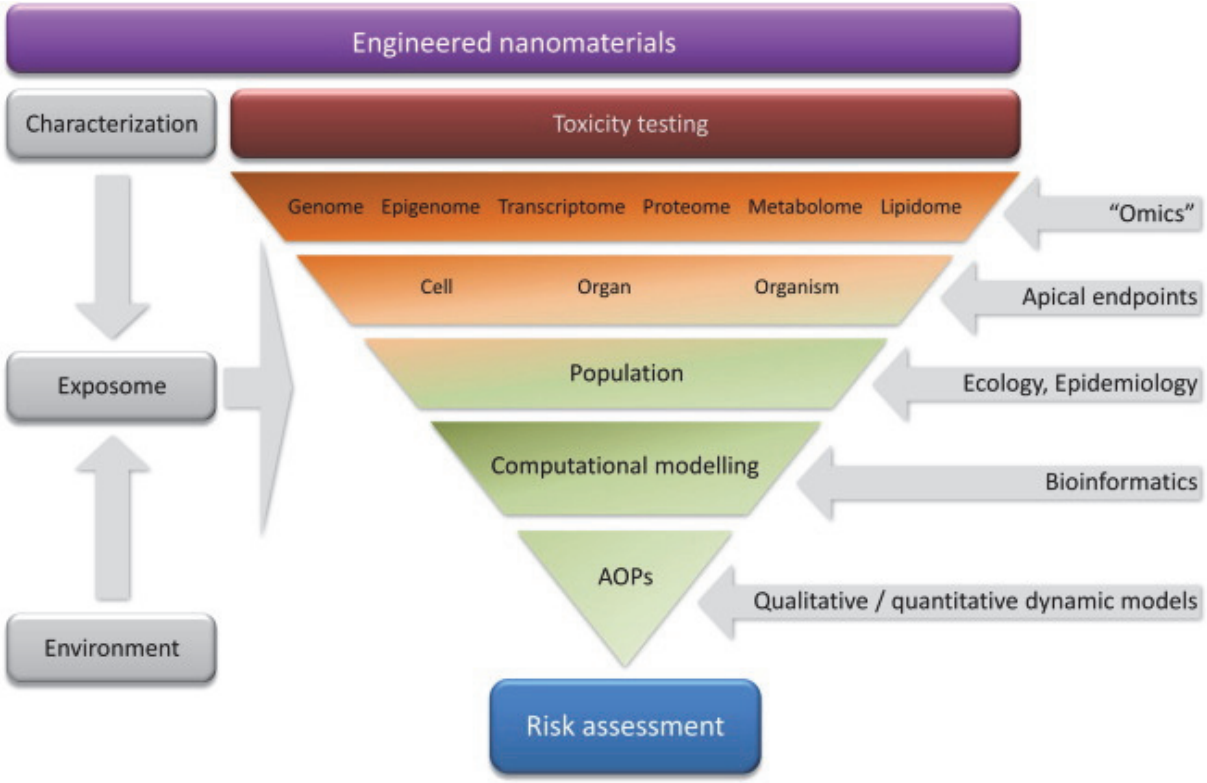


Figure 13. Systems toxicology framework. The schematic figure shows how omics-enabled modelling of ENM-induced perturbations may inform so-called adverse outcome pathways or AOPs, to support risk assessment. From: Costa and Fadeel (2016).

Analysis of one omics 'data layer' at a time (eg., proteomics or transcriptomics) is currently relatively simple and provides information about the toxicant-induced molecular changes in experimental models (*in vitro* and *in vivo*). Several commercial and non-commercial bioinformatics tools for pathway enrichment analysis, upstream regulator prediction and versatile visualization of differentially expressed/abundant genes/miRNA/proteins exits. Depending on the system, omics can also allow for the identification of molecular pathways that are perturbed and predict initial molecular events leading to adverse outcomes. It should be noted however, that although progress in omics technologies has been extremely rapid during recent years and while these approaches are generating impressive amounts of high-quality data, each method comes with its own advantages and limitations. Due to relatively high cost, requirement of highly specialized and expensive equipment, and slow analysis speed per sample, only a few biological replicates are usually analyzed; thus, omics data analysis is hardly 'high-throughput'. Moreover, it can be challenging to separate toxicant-induced, biologically relevant changes from artificially induced small differences unless research design, analysis strategy and quality issues are strictly controlled (Hartung et al., 2017).

Transcriptomics studies in nanosafety research

Microarray analysis of gene expression changes have been recently used in the evaluation of reliability and suitability of experimental toxicology models in nanotoxicology research. In the study by Kinaret et al. (2017a), the authors examined the comparability between two alternative *in vivo* exposure methods, i.,e., inhalation *versus* pharyngeal aspiration exposure, used for hazard assessment of ENMs. Inhalation and low-dose aspiration exposure to rod-shaped multi-walled CNTs elicited very similar pulmonary inflammation in mice. Moreover, both exposure methods induced similar alterations in gene expression with 154 (56.4%) differentially expressed, overlapping genes in the microarray analysis. Of all differentially expressed genes, up to 80% of the activated biological functions were shared according to pathway enrichment analyses. The data suggested that oropharyngeal aspiration could be a valid alternative to the expensive and laborious inhalation protocol for the hazard assessment of ENMs. In another study (Kinaret et al., 2017b), transcriptomic responses of the human THP-1 cell line and lung tissues of mice were investigated after exposure to several carbon-based nanomaterials. Only a minor overlap between the differentially expressed genes in the *in vitro* and *in vivo* experiments was observed, suggesting

specific transcriptional programs in these models. However, when the effects of the ENMs were investigated at the level of significantly altered molecular functions, a broader picture of substantial commonality emerged. The results implied that *in vitro* exposures could recapitulate the complex molecular processes that were altered *in vivo* (Kinaret et al., 2017b).

Gene expression profiling has developed rapidly in recent years with the advent of deep sequencing technologies such as RNA sequencing (RNA-seq). In a recent study performed at IMM, Feliu et al. (2015) examined the cellular effects of exposure to dendrimers by RNA-seq and showed that amino-functionalized dendrimers (PAMAM-NH_2) are capable of triggering downregulation of cell cycle-related genes in primary human bronchial epithelial cells at doses that did not elicit acute cytotoxicity. Upstream regulator analysis implicated NF-κB as a putative transcriptional regulator, and subsequent cell-based assays confirmed that PAMAM-NH_2 caused NF-κB-dependent cell cycle arrest. These results demonstrated the feasibility of applying systems biology approaches to predict cellular responses to ENMs and highlight the importance of using relevant cell models. This work also highlights the importance of addressing low-dose effects of ENMs. Mortimer et al. (2018) applied RNA-seq to study the impact of carbonaceous and boron nitride-based nanomaterials at non-growth-inhibitory concentrations (10 mg/L), using *Pseudomonas aeruginosa* as a model system. Mitchell et al. (2016) investigated whether cells respond differently in relation to differences in the cellular burden of ENMs. To this end, they applied single-cell RNA-seq to alveolar epithelial cells carrying defined loads of aminated or carboxylated quantum dots (QDs). Interestingly, cells carrying lower loads responded with multiple cellular ‘strategies’, which were nonetheless coherent and unique to each QD type, while cells carrying higher loads responded more uniformly across different QD types. Taken together, these studies testify to the considerable sensitivity of RNA-seq and show how transcriptomics approaches can unlock cellular responses to ENMs also at the single-cell level.

Prediction of nanotoxicity using omics data

Systems toxicology has opened completely new avenues for prediction of ENM safety. Huge amounts of redundant information is usually produced in omics experiments, and in order to identify the relevant molecular features (signatures), one needs to be able to isolate the relevant information while taking into account the high levels of statistical

dependency between the variables. In this context, the group of features that best predicts the safety of ENMs might not be composed only of elements derived from one data layer, but also by features with a combinatorial effect derived from multiple layers of complex data. The EU-funded project, FP7-NANOSOLUTIONS, aimed at generating a computer algorithm capable of predicting the safety of ENMs based on a minimal but most informative set of features selected across multiple data layers. Based on integration of data from 5 data layers (i.e., microarray-based transcriptomics, RNA-seq, miRNA profiling, proteomics, and bio-corona profiling) as well as the physicochemical properties for 31 different ENMs, a classifier algorithm, composed only of 16 hazard-associated features, was generated capable of predicting with a high accuracy ENM toxicity (Fadeel et al., 2018). In another study, Serra et al. (2018) could show that meaningful predictions regarding the association between specific ENMs and human diseases could be obtained based on mode-of-action similarities at the transcriptional level. Computational predictive approaches represent a major leap forward and may provide more rapid hazard classification based on relatively small numbers of toxicity studies.

Concluding remarks

To conclude, omics studies and the computational analysis of such data hold great promise in nanotoxicology. Remaining challenges relate to how ENM-induced network perturbations are to be linked to toxicological phenotypes, which will require a deeper understanding of the molecular mechanisms that give rise to adverse responses. Integration of systems level data derived from *in vitro* and *in vivo* experiments with computational modelling will be critically important in nanosafety research. Moreover, multi-omics studies that integrate several layers of data at the transcriptome, proteome, and metabolome level over time may offer the best ability to develop reliable mechanism-based analysis strategies that are applicable in both effects monitoring and in a risk assessment framework.

References

Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol 2014; 32:760-72.

Collins FS, Gray GM, Bucher JR. Toxicology. Transforming environmental health protection. Science. 2008;319(5865):906-7.

Costa PM, Fadeel B. Emerging systems biology approaches in nanotoxicology: towards a mechanism-based understanding of nanomaterial hazard and risk. Toxicol Appl Pharmacol. 2016;299:101-11.

Fadeel B. Systems biology in nanosafety research. Nanomedicine (Lond). 2015;10(7):1039-41.

Fadeel B, Farcal L, Hardy B, Vázquez-Campos S, Hristozov D, Marcomini A, Lynch I, Valsami-Jones E, Alenius H, Savolainen K. Advanced tools for the safety assessment of nanomaterials. Nat Nanotechnol. 2018;13(7):537-543.

Feliu N, Kohonen P, Ji J, Zhang Y, Karlsson HL, Palmberg L, Nyström A, Fadeel B. Next-generation sequencing reveals low-dose effects of cationic dendrimers in primary human bronchial epithelial cells. ACS Nano 2015; 9:146-63.

Hartung T, FitzGerald RE, Jennings P, Mirams GR, Peitsch MC, Rostami-Hodjegan A, Shah I, Wilks MF, Sturla SJ. Systems toxicology: real world applications and opportunities. Chem Res Toxicol 2017; 30:870-82.

Kinaret P, Ilves M, Fortino V, Rydman E, Karisola P, Lahde A, Koivisto J, Jokiniemi J, Wolff H, Savolainen K, Greco D, Alenius H. Inhalation and oropharyngeal aspiration exposure to rod-like carbon nanotubes induce similar airway inflammation and biological responses in mouse lungs. ACS Nano 2017a; 11:291-303.

Kinaret P, Marwah V, Fortino V, Ilves M, Wolff H, Ruokolainen L, Auvinen P, Savolainen K, Alenius H, Greco D. Network analysis reveals similar transcriptomic responses to intrinsic properties of carbon nanomaterials in vitro and in vivo. ACS Nano 2017b; 11:3786-96.

Mitchell HD, Markillie LM, Chrisler WB, Gaffrey MJ, Hu D, Szymanski CJ, Xie Y, Melby ES, Dohnalkova A, Taylor RC, Grate EK, Cooley SK, McDermott JE, Heredia-Langner A, Orr G. Cells respond to distinct nanoparticle properties with multiple strategies as revealed by single-cell RNA-Seq. ACS Nano. 2016;10(11):10173-10185.

Mortimer M, Devarajan N, Li D, Holden PA. Multiwall carbon nanotubes induce more pronounced transcriptomic responses in *Pseudomonas aeruginosa* PG201 than graphene, exfoliated boron nitride, or carbon black. ACS Nano. 2018;12(3):2728-2740.

National Research Council. 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy. Washington, DC: The National Academies Press. https://doi.org/10.17226/11970.

Serra A, Letunic I, Fortino V, Handy RD, Fadeel B, Tagliaferri R, Greco D. insideNANO: a systems biology framework to contextualize the mechanism-of-action of engineered nanomaterials. Sci Rep. 2018 [in press].

Sturla SJ, Boobis AR, FitzGerald RE, Hoeng J, Kavlock RJ, Schirmer K, Whelan M, Wilks MF, Peitsch MC. Systems toxicology: from basic research to risk assessment. Chem Res Toxicol. 2014;27(3):314-29.

5.4. Structure-Activity Relationship Modeling

Structure-activity relationship (SAR) modeling of toxicological effects aims to associate the chemical structure with modes of binding, reactivity and biological activity, and the linkage of related measurements to the induction of toxicity and pathological effects (Guha et al., 2012; Cherkasov et al., 2014; Tsiliki et al., 2015; Garcia-Serna et al., 2015). So-called (quantitative) structure activity-relationship [(Q)SAR] methods are currently among the most commonly used animal-free *in silico* methods for predicting chemical toxicity, and also one of the most widely accepted starting points for grouping and read across to fill data gaps during risk assessment or registration of novel materials under the REACH legislation. To facilitate practical application of (Q)SAR approaches in regulatory contexts by governments and industry and to improve their regulatory acceptance, the OECD (Q)SAR project, in collaboration with the European Union, has developed various outcomes such as guidance documents, and the QSAR Toolbox (http://www.qsartoolbox.org). In the pharmaceutical industry, (Q)SAR approaches serve extensively to remove "structural alerts-positive" compounds early in the discovery process, serving as one important means of minimizing later stage clinical failure of drugs (Maertens et al., 2014; Garcia-Serna et al., 2015). However, (Q)SAR methods developed for pharmaceuticals are largely targeted towards identifying biological activity and lack the accuracy for defining non-toxicity, which is more relevant for the testing of industrial chemicals and ENMs. In addition, pharmaceuticals cover a relatively narrow chemical space and are usually highly specific in their toxic mechanisms, while industrial substances, in particular nanomaterials, are more complex and varied, and thus place higher demands on (Q)SARs (Maertens et al., 2014).

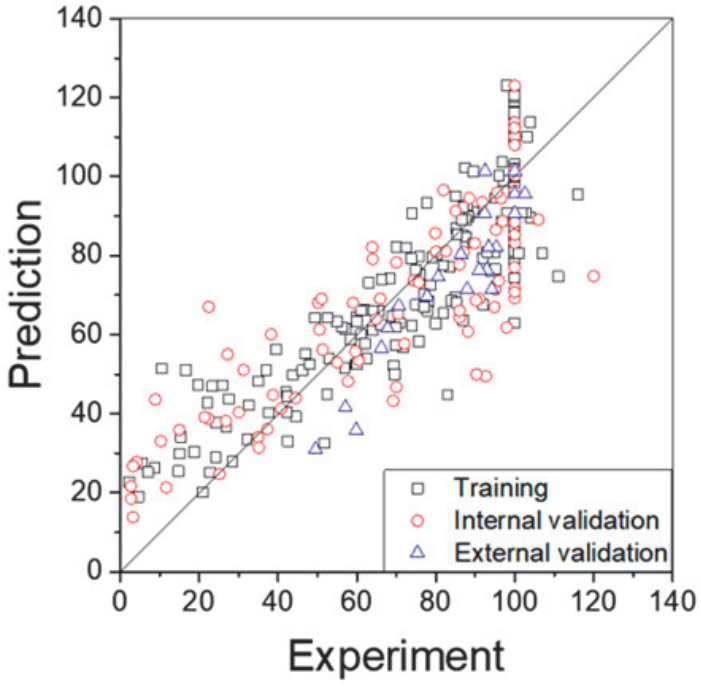
(Q)SARs can be based on largely any type of regression or classification method, such as multiple linear regression, partial least squares, decision trees, linear discriminant analysis or artificial neural networks (Oksel et al., 2016). However, the physicochemical properties of ENMs are complex and highly context-dependent, and have been explored to a lesser extent compared to chemicals (Lynch et al., 2014). Several reviews on the subject have identified a lack of high quality, systematically gathered nanotoxicological datasets, large enough to enable robust (Q)SAR modeling (Lynch et al., 2014; Oksel et al., 2015; Tantra et al., 2015; Winkler et al., 2016). A few major challenges have been identified and can be summarized as a lack of common experimental guidelines (SOPs) to characterize and assess interlinked physicochemical properties in parallel with the toxicity of ENMs, as well as a lack of harmonized data

management and storage strategies (Lynch et al., 2014; Tantra et al., 2015). However, efforts to remedy this situation are being made, and a large dataset containing information on the physicochemical properties for more than 300 ENMs (the NanoWiki dataset) is now available through the EU-funded eNANOMAPPER project (Jeliazkova et al., 2015). As demonstrated for medicinal chemistry, the growth of curated chemical databases has been key to the advancement of (Q)SAR modeling (Cherkasov et al., 2014). Nano-(Q)SAR modelling, on the other hand, is still in its infancy, though advances have been made and (Q)SAR has been applied to identify various types of physicochemical properties of concern and as a basis for 'safe design' of CNTs (Tantra et al., 2014; Oksel et al., 2015; Fourches et al., 2016). However, the generalizability of the models is limited in the absence of proper model validation, due to the lack of sufficiently large data sets, and because of poor understanding of the underlying mechanisms of toxicity (Oksel et al., 2015). As a consequence, innovative application and integration of concepts and tools from other fields are being exploited. For example, Oksel et al. (2016) showed that decision tree-based SAR models are more transparent, can deal with small data sets, and provide a robust basis for interpretation in terms of toxicological mechanisms. The method is strongly focused on the selection of key descriptors and, in fact, a central notion in the field is that the interpretability of (Q)SAR models is largely driven by the choice of descriptors (Fujita et al., 2016). However, even if (Q)SAR models can provide insight into the mechanisms of action, it must always be recognized that the correlations do not guarantee causality (Cherkasov et al., 2014).

The complexity of the physicochemical properties of ENMs and their correlation with toxicity have triggered an interest in the use of biological and molecular effects *in vitro* to predict toxic effects *in vivo* (Nel et al., 2012). Linking structure through molecular modeling of data from novel biological data-rich techniques, such as high-throughput screening (HTS) and omics, together with well-developed and advanced *in vitro* models is increasingly seen as a realistic approach to risk characterization of nanomaterials (Nel et al., 2012; Grafström et al., 2015; Collins et al., 2016). However, these approaches rely heavily on the existence of well-characterized, systematically varied libraries of ENMs representative of different types of toxicity (Banares et al., 2017). Trinh et al (2018) developed a nano-QSAR model to predict the cytotoxicity of multi-walled CNTs to human lung cells using data for 20 MWCNTs extracted from the Safe & Sustainable Nanotechnology database (www.s2nano.org). The data originated from 10 different research articles. However, there are also

efforts to establish libraries, for instance, the representative nanomaterials housed by the Joint Research Centre (JRC) of the European Commission, and sets of ENMs developed within specific projects, such as the EU-funded project, FP7-NANOSOLUTIONS. The JRC Nanomaterials Repository was initially set up to support the testing programme of the OECD WPMN and thus hosts the principal nanomaterials that have been examined by the OECD. More recently other ENMs have been added thus widening the types of materials hosted. The NANOSOLUTIONS set of ENMs represents a good example of a systematically varied library, where a set of 8 core nanomaterials of different chemical compostion and their aminated, PEGylated, and carboxylated variants were tested in a wide array of toxicological assays, using both *in vitro* and *in vivo* model systems.

$$\boldsymbol{Cell\ viability = C_0 + C_1 \times DCW}$$



$$\boldsymbol{DCW = f(PChem^{MWCNT}, exposure\ condition)}$$

Figure 14. Quasi-SMILES-based nano-QSAR model to predict the cytotoxicity of multiwalled carbon nanotubes to lung cells. From: Trinh et al. Chem Res Toxicol. 2018.

In relation to chemical toxicity, integrated approaches combining (Q)SAR and omics methods in chemical-biological modelling, with each type of data weighted according to reliability and expected predictive ability, have begun to materialize. For example, Low et al. (2013) recently showed that

transcriptomics data and chemical descriptors combined in chemical-biological read across (CBRA)-based hazard classification consistently exhibited high classification accuracy and applicability across diverse chemicals. The method also highlights key biological and chemical features for further mechanistic interpretation. Omics data were also used recently to predict cell association of gold nanoparticles based on their protein corona profiles assessed by proteomic techniques, and was shown to increase the predictivity as compared to using only physicochemical characteristics (Walkey et al., 2014; Liu et al., 2015). However, it has been argued that novel biology-based modeling approaches face the danger of focusing too narrowly on physicochemical aspects, while reducing complex biological phenomena into simplistic numerical values (Cherkasov et al., 2014). Initiatives to overcome these hurdles are ongoing, e.g., within the EU-funded project SmartNanoTox, with the objective to link the potential for adverse effects to nanoscale physicochemical properties, through so called mechanism-aware (Q)SAR modeling, i.e. linkage of physicochemical properties with molecular initiating and key events (MIE/KE) established in adverse outcome pathways (AOP). Alongside experimental characterization, the project is strongly focused on modeling of chemical interactions based on computational chemistry and may be able to contribute to the identification of key molecular and physicochemical characteristics that determine toxicity. Computational simulations model molecular interactions between chemical and biological structures, e.g., between nanoparticle surfaces and cellular membranes, and predict behavior on the atomic and molecular dynamics/mechanics level (Brandt et al., 2016; Lopez et al., 2017). Such modelling may lead to the development of databases containing predicted information on bio-nano interactions, useful as descriptors for (Q)SAR modeling. The databases could be similar in design as the various molecular interaction databases established to date, examples being DNA-protein interaction and microRNA-target prediction databases (Orchard et al., 2012). Furthermore, pathway-based descriptors may overcome unnecessary numerical simplification of biological mechanisms. For example, a recently published genomics-based description of chemical toxicological responses broadly captures and accurately score for liver toxicity on both cellular and organismal level, while at the same time retaining mechanistic information and precision (Kohonen et al., 2017). Interestingly, preliminary analyses of the description, referred to as the Predictive Toxicogenomics Space (PTGS), have indicated that the tool covers most of the responses to ENMs seen to date, including oxidative stress, apoptosis, cell cycle changes, receptor-mediated responses, and DNA repair (Kohonen et al., unpublished results).

Concluding remarks

(Q)SAR methods for ENMs are beginning to emerge, and innovative strategies towards inclusion of biological data (e.g., omics data) as well as integration with computational chemistry and the AOP concept are increasingly explored. Better data management procedures and Tox21-inspired data generation work flows, such as the one described in the chapter on HTS, particularly for large libraries of well-characterized ENMs, will allow for the development of curated databases. In addition, progress in the field, especially with regard to implementation of commonly agreed upon grouping and read across strategies, relies heavily on efficient communication and collaboration between computational, experimental, and risk assessment experts (Cherkasov et al., 2014; Dekkers et al., 2016).

References

Bañares MA, et al. CompNanoTox2015: novel perspectives from a European conference on computational nanotoxicology on predictive nanotoxicology. Nanotoxicology. 2017;11(7):839-845.

Brandt et al. Reactive wetting properties of TiO_2 nanoparticles predicted by ab initio molecular dynamics simulations. Nanoscale. 2016;8(27):13385-98.

Cherkasov et al. QSAR modeling: Where have you been? Where are you going to? J. Med. Chem., 2014, 57 (12), pp 4977–5010

Collins, A.R., et al., High throughput toxicity screening and intracellular detection of nanomaterials. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology, 2017. 9(1): p. e1413.

Dekkers S, et al. Towards a nanospecific approach for risk assessment. Regul Toxicol Pharmacol. 2016;80:46-59.

Fourches, et al. Computer-aided design of carbon nanotubes with the desired bioactivity and safety profiles. Nanotoxicology. 2016;10(3):374-83.

Fujita et al. Understanding the roles of the “Two QSARs”. J. Chem. Inf. Model. 2016, 56, 269−274.

Gajewicz et al. Towards understanding mechanisms governing cytotoxicity of metal oxides nanoparticles: Hints from nano-QSAR studies. Nanotoxicology, 2015; 9(3): 313-325.

Garcia-Serna et al. Large-scale predictive drug safety: from structural alerts to biological mechanisms. Chem. Res. Toxicol., 2015, 28 (10), pp 1875–1887.

Grafström, R., et al., Toward the replacement of animal experiments through the bioinformatics-driven analysis of 'omics' data from human cell cultures. Altern Lab Anim, 2015. 43(5): p. 325-32.

Guha et al. A survey of quantitative descriptions of molecular structure. Curr Top Med Chem. 2012; 12(18): 1946–1956.

Jeliazkova N, et al. The eNanoMapper database for nanomaterial safety information. Beilstein J Nanotechnol. 2015 Jul 27;6:1609-34.

Kohonen P, et al. A transcriptomics data-driven gene space accurately predicts liver cytopathology and drug-induced liver injury. Nat Commun. 2017;8:15932.

Liu et al. Prediction of nanoparticles-cell association based on corona proteins and physicochemical properties. Nanoscale. 2015 Jun 7;7(21):9664-75.

Low et al. Integrative chemical-biological read-across approach for chemical hazard classification. Chem Res Toxicol. 2013 Aug 19; 26(8): 10.1021/tx400110f.

Lopez et al. 2017. Multiscale Modelling of Bionano Interface. In: *Modelling the Toxicity of Nanoparticles*. Advances in Experimental Medicine and Biology, Vol. 947, pp. 173-206.

Lynch et al. A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs. Nano Today 2014; 9(3): 266-270.

Maertens et al. Green toxicology. ALTEX. 2014;31(3):243-9.

Nel, A., et al., Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high throughput screening. Accounts of chemical research, 2013. 46(3): p. 607-621.

Oksel et al. (Q)SAR modelling of nanomaterial toxicity: a critical review. Particuology 21 (2015) 1–19

Oksel et al. Accurate and interpretable nanoSAR models from genetic programming-based decision tree construction approaches. Nanotoxicology, 2016; 10(7): 1001–1012

Orchard et al. Molecular interaction databases. Proteomics, 2012: 12(10)1656–1662.

Tantra et al, Nano(Q)SAR: challenges, pitfalls and perspectives. Nanotoxicology, 2015; 9(5): 636–642.

Trinh TX, Choi JS, Jeon H, Byun HG, Yoon TH, Kim J. Quasi-SMILES-based nano-quantitative structure-activity relationship model to predict the cytotoxicity of multiwalled carbon nanotubes to human lung cells. Chem Res Toxicol. 2018;31(3):183-190.

Tsiliki et al. RRegrs: an R package for computer-aided model selection with multiple regression models. J Cheminform (2015) 7:46.

Walkey et al. Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. ACS Nano. 2014 Mar 25;8(3):2439-55.

Winkler. Recent advances, and unresolved issues, in the application of computational modelling to the prediction of the biological effects of nanomaterials. Toxicology and Applied Pharmacology 299 (2016) 96–100.

5.5. Adverse Outcome Pathways (AOPs)

The use of adverse outcome pathways (AOP) may enable and promote the incorporation of mechanistic data in risk assessment AOPs may serve a number of purposes, including the establishment of (quantitative) structure-activity relationships, and the elaboration of prioritization strategies (Vinken, 2013). The AOP concept is expected to support the 21st century paradigm shift aimed at reducing the reliance on *in vivo* toxicity testing and increasing human relevance and predictivity of alternative methods, typically from analysis and modeling of results obtained from human cell-based tests rather than animal tests (Burden et al., 2015).

An AOP is a scheme that depicts the most central physiological events leading to disease, and uses existing knowledge to causally connect a molecular initiating event (MIE) to key events (KE), associative events (AE), and an adverse outcome (AO) (Ankley et al., 2010). The different events can be linked to toxicological tests and assays, which provide a basis for integrated testing strategies (ITS) predictive of adverse outcome (Burden et al., 2015). To ensure accurate prediction, the events need to be critical and essential (but not necessarily sufficient) for the final AO, and supported by evidence-based causal relationships, i.e., key event relationships (KERs) (Kleinstreuer et al., 2016). This structured gathering of data can help identify knowledge gaps and guide research towards filling them. In addition, since KEs can be shared by different AOPs, and one MIE can lead to multiple AOs and *vice versa*, AOPs in general form networks, which are expected to inform risk assessment on several levels, including test method prioritization, based on central or 'nodal' KEs involved in several AOPs (Villeneuve et al., 2014; Kleinstreuer et al., 2016). Inflammation is a common response to a variety of stressors, including xenobiotics, and as such it is anticipated that inflammation would emerge as a highly connected node within an AOP network. Recently, a proposal for the interconnection of previously unrelated AOPs by using a set of 'hub' KEs related to inflammation was put forward (Villeneuve et al., 2018).

The AOPs are related to the concept of mode-of-action (MoA), which has governed risk assessment during the last decades, as a way of utilizing mechanistic data. However, an important difference between AOPs and MoAs lies in the relationship with the perturbant; MoAs tend to describe details specific to a particular perturbant or perturbant class, while AOPs describe the perturbed biological pathways and are (ideally) chemical

agnostic (Kleinstreuer et al., 2016). This move towards better descriptions of the biological responses and toxicological pathways enables analysis of biological similarity, in order to aid read across based on biological data to complement chemical structure-based read across (Zhu et al., 2016). This also means that the qualitative mechanistic knowledge captured in AOPs, developed based on chemically induced toxicity, serve equally well for describing toxicity of ENMs, which seems to result in the same toxicity as chemicals, though there is still a lack of detail and understanding of the MIE (Gerloff et al., 2017). It has been argued that the MIE through which nanomaterials initiate toxicity is less specific when compared to chemicals, which often act through receptor binding or other specific molecular interactions (Gerloff et al., 2017). The toxic effects of ENMs are commonly thought to be mechanical or physical, although some synergistic specific effects could be expected, such as those caused by ion leaching. An example is the Trojan horse effect, in which nanoparticles release high levels of toxic ions only upon internalization, causing a specific ion-related effect inside the cell, which is, however, initiated through a non-specific uptake of the nanomaterial (Cronholm et al., 2013). In fact, Gerloff et al. (2017) suggested that MIEs may not be as relevant for nanomaterials as for chemicals and that the first KE could be assigned as simply an initial KE. However, for some ENMs, a more specific mechanism of action could be envisioned – some nanoparticles are apparently able to engage the signaling machinery of cells in a manner that is similar to chemicals or endogenous signaling molecules (reviewed in: Gallud and Fadeel, 2015).

Development and enrichment of AOPs

Development of AOPs involves extracting information across diverse perturbant-specific studies. Such efforts should not be a nano-specific exercise, although some valuable efforts have been made towards building AOPs based on nano-specific information. Vietti et al. (2016) used literature focused exclusively on carbon nanotubes (CNTs), to map the current knowledge on molecular key players (biomarkers for KEs) involved in the development of CNT-induced lung fibrosis, with the aim of building an AOP for the disease. Similarly, Labib et al. (2016) used transcriptomics data from CNT-exposed lung fibrosis-developing mice, to build a putative AOP and map biological pathways identified through bioinformatics analysis to each MIE, KE and AE, demonstrating the usefulness of omics data for deriving pathway-based biomarkers. These approaches represent a starting point, but the added benefit of looking into knowledge from other fibrogenic materials/chemicals to fill gaps and to generate a wider suite of pro-fibrotic biomarkers is currently being explored (Clippinger et al., 2016). Another approach towards generating AOPs applicable to ENMs, while

also taking also chemical-induced information into consideration, was recently presented in relation to liver damage (Gerloff et al., 2017). A number of AOPs for chemical-induced liver effects are currently being elborated within the OECD, and although the MIE may be different, their value with regard to the downstream effects has been recognized, leading to the development of an AOP for liver inflammation in relation to the toxic effects of SiO_2 and TiO_2 nanoparticles. The AOP was further merged with an AOP for liver fibrosis developed based on knowledge from chemical toxicity. The integration of the two AOPs was guided by overlapping downstream events and indicated that the nanoparticles can be expected to induce liver fibrosis *via* KEs and KERs already known for chemically induced fibrosis. The authors suggested that the differences between nanomaterial- and chemically-induced adversities are primarily related to differences in toxicokinetics and the nature of the initial KEs (Gerloff et al., 2017).

To facilitate the development of AOPs and to promote transparency, the AOP Knowledgebase (https://aopkb.org) has been established comprising tools assisting development and an AOP Wiki for open access publication of AOPs. The AOP Wiki allows users to cooperate in documenting and evaluating diverse information underlying AOPs and support the development of new approaches for the validation of alternative testing methods. The final goal is to enable AOP-based ranking and prioritization, and/or classification based on MIEs and KEs (in cellular models) (Langley et al., 2015). Thus, AOPs can be seen as a foundation for merging Integrated Approaches to Testing and Assessment (IATA) with data-driven regulatory decision making (Tollefsen et al., 2014; Nel and Malloy, 2017).
Recent efforts have been made to enrich AOP descriptions by utilizing omics data (Nymark et al., 2018a, *idem*, 2018b). Hence, Nymark et al. (2018b) devised a workflow that integrated diverse types of toxicology data into a novel AOP scheme for pulmonary fibrosis. Ultimately, a network of functional elements coupled 64 pulmonary fibrosis-associated genes into a novel, open-source AOP-linked molecular pathway, which is available for comments and improvements in at the AOP Wiki. This approach may provide a useful approach to constructing novel AOP descriptions as well as to enrich existing AOP descriptions. In conclusion, AOPs enable integration of diverse data and facilitate the use of data streams often not employed by risk assessors, including information from *in silico* models, *in vitro* assays, and short-term *in vivo* tests (Ankley and Edwards, 2018). On the other hand, the AOP concept also profits from the advances in molecular toxicology and bioinformatics aimed at mechanistic analyses, which have been difficult to use for the prediction of adverse outcomes

(Grafström et al., 2015). Systems biology-based assays and strategies relying upon quantitative mechanistic information are increasingly envisaged as cornerstones of future safety evaluations of nanomaterials. Large-scale chemical- and drug toxicity-related consortia and projects, e.g., Tox21, ToxCast and SEURAT/EUToxRisk, address thesse issues by complementing the traditional structure-based analysis with the development of "new approach methodologies" for safety prediction inspired by the AOP concept, including transcriptomics assays (Grafström et al., 2015; Langley et al., 2015; Collins et al., 2017; ECHA, 2017). The value of the information generated in these parallel fields of toxicology should not be underestimated in the area of nanosafety assessment.

"Systems biology-based assays and strategies relying upon quantitative mechanistic information are increasingly envisaged as cornerstones of future safety evaluations of nanomaterials."

References

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 2010;29(3):730-41.

Ankley GT, Edwards SW. The adverse outcome pathway: a multifaceted framework supporting 21st century toxicology. Curr Opin Toxicol. 2018;9:1-7.

Burden N, Sewell F, Andersen ME, Boobis A, Chipman JK, Cronin MT, Hutchinson TH, Kimber I, Whelan M. Adverse outcome pathways can drive non-animal approaches for safety assessment. J Appl Toxicol. 2015;35(9):971-5.

Clippinger AJ, et al. Expert consensus on an in vitro approach to assess pulmonary fibrogenic potential of aerosolized nanomaterials. Arch Toxicol. 2016;90(7):1769-83.

Cronholm P, Karlsson HL, Hedberg J, Lowe TA, Winnberg L, Elihn K, Wallinder IO, Möller L. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small. 2013;9(7):970-82.

Gallud A, Fadeel B. Keeping it small: towards a molecular definition of nanotoxicology. Eur J Nanomedicine, 2015;7(3):143-151.

Grafström, R., et al., Toward the replacement of animal experiments through the bioinformatics-driven analysis of 'omics' data from human cell cultures. Altern Lab Anim, 2015. 43(5): p. 325-32.

Gerloff K, Landesmann B, Worth A, Munn S, Palosaari T, Whelan M. The adverse outcome pathway approach in nanotoxicology. Comput. Toxicol., 2017;1:3-11.

Kleinstreuer NC, Sullivan K, Allen D, Edwards S, Mendrick DL, Embry M, Matheson J, Rowlands JC, Munn S, Maull E, Casey W. Adverse outcome pathways: From research to regulation scientific workshop report. Regul Toxicol Pharmacol. 2016;76:39-50.

Labib S, Williams A, Yauk CL, Nikota JK, Wallin H, Vogel U, Halappanavar S. Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part Fibre Toxicol. 2016;13:15.

Langley, G. et al. Lessons from toxicology: developing a 21st-century paradigm for medical research. Environ Health Perspect. 123, A268-72 (2015).

Nel AE, Malloy TF, Policy reforms to update chemical safety testing. Science, 2017; 355(6329): 1016-1018.

Nymark P, Kohonen P, Hongisto V, Grafström RC. Toxic and genomic influences of inhaled nanomaterials as a basis for predicting adverse outcome. Ann Am Thorac Soc. 2018a;15(Suppl. 2):S91-S97.

Nymark P, Rieswijk L, Ehrhart F, Jeliazkova N, Tsiliki G, Sarimveis H, Evelo CT, Hongisto V, Kohonen P, Willighagen E, Grafström RC. A data fusion pipeline for generating and enriching adverse outcome pathway descriptions. Toxicol Sci. 2018b;162(1):264-275.

Tollefsen KE, Scholz S, Cronin MT, Edwards SW, de Knecht J, Crofton K, Garcia-Reyero N, Hartung T, Worth A, Patlewicz G. Applying adverse outcome pathways (AOPs) to support integrated approaches to testing and assessment (IATA). Regul Toxicol Pharmacol. 2014;70(3):629-40.

Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA, Landesmann B, Lettieri T, Munn S, Nepelska M, Ottinger MA, Vergauwen L, Whelan M. Adverse outcome pathway development II: best practices. Toxicol Sci. 2014;142(2):321-30.

Villeneuve DL, et al. Representing the process of inflammation as key events in adverse outcome pathways. Toxicol Sci. 2018;163(2):346-352.

Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. Toxicology. 2013;312:158-65.

Zhu H, Bouhifd M, Donley E, Egnash L, Kleinstreuer N, Kroese ED, Liu Z, Luechtefeld T, Palmer J, Pamies D, Shen J, Strauss V, Wu S, Hartung T. Supporting read-across using biological data. ALTEX. 2016;33(2):167-82.

5.6. Data Management: Supporting Nano Risk Governance

Good data management in science is key to knowledge discovery and innovation (Wilkinson et al., 2016). The increasingly multi-disciplinary field of nanosafety constitutes a challenge with regard to data management, mainly due to inherently complex nanomaterial characteristics and the multi-disciplinary nature of the data generated in the field. Comparison and integration of information between multi-disciplinary projects aimed at evaluating nanomaterial safety is therefore increasingly recognized as being hindered from non-sustainable storage and annotation of the data in diverse manners. Several collaborative initiatives to harmonize and eventually to standardize nanosafety data collection and interoperability have emerged. The EU-funded project FP7-eNANOMAPPER approached the challenge by establishing a computational infrastructure for nanotoxicological data management, based on extensive requirement analysis, review of existing solutions and direct interactions with scientists in the field (Jeliazkova et al., 2015; Hastings et al., 2015). The project developed an open source, interactive and searchable database, a nanosafety-specific ontology, and integrated data analysis services, all available online (http://enanomapper.net). In parallel, the Nanomaterial Data Curation Initiative (NDCI), a project of the National Cancer Informatics Program Nanotechnology working group in the United States has explored critical aspects of data curation and has undertaken the role of addressing complex topics like uncertainty, reproducibility, and interoperability and providing solutions to complex data integration, largely taking examples from the bioinformatics community (Hendren et al., 2015).

Data *templates* are one of the most crucial components of harmonized data gathering. The NDCI has suggested a shift towards approaches requiring authors/investigators to add their data into public repositories as the most sustainable option, based on the successful implementation of such practice within the bioinformatics community (Powers et al., 2015). In bioinformatics, this way of operating was implemented soon after the rise of the omics technologies, to facilitate the establishment of databases and public repositories and enable the development of data analysis tools (Brazma et al., 2001). Today, omics data is commonly deposited in online repositories upon publication as a requirement by most high impact journals, enabling widespread reanalysis and recycling of the data. Data can also be deposited prior to publication and held non-public, but shared with partners with whom agreements have been made. This applies also to omics data sets generated within the nanosafety community; however, the lack of links to associated nanomaterial characterization data still hinders high level quality analysis. Several projects within the nanosafety

community have tackled this challenge by enforcing harmonized templates to be used by all the participating partners. Recently, the efforts have begun to converge at a higher level and by the end of the large EU-funded project FP7-NANOREG in March 2017, a set of data templates recommended for use within the nanosafety community had been published (http://www.nanoreg.eu). These Excel-based templates follow the logic of the Investigation Study Assay (ISA) framework, which supports interoperability and promotes the growth of a culture of so-called 'data commoning' (Sansone et al., 2012). In the nanosafety community, a need for an added focus on material characteristics was recognized and an ISA-TAB-Nano extension was developed to include a material description sheet (Thomas et al., 2013). Although the NANOREG templates were distributed and used in an Excel-based format, further developments have enabled the conversion of data from one format to another directly in the eNANOMAPPER database (Jeliazkova et al., 2015). In 2017, the complete NANOREG dataset was included in the eNANOMAPPER database.

In addition to data templates, a commonly agreed upon terminology or *ontology* plays an equally important role. A well-described ontology lies at the heart of linking and integrating data, as demonstrated by the bioinformatics community and its successful use of the Gene Ontology, which has enabled novel means for discovery through integration of widely diverse bioscience data across multiple technologies and species (Ashburner et al., 2000). The first initiative to generate a nano-specific ontology was undertaken in the cancer nanotechnology research field in the United States and this resulted in the 'nanoparticle ontology', largely focused on the characteristics of nanomaterials involved in cancer research (Thomas et al., 2011). Subsequently, eNANOMAPPER adopted and extended the effort, by integrating previously developed ontologies from other disciplines to establish a wider nanosafety focused ontology. The innovative approach to re-use and seamlessly combine existing ontologies previously developed by experts in nanosafety-related fields enabled the generation of a highly dynamic ontology that is guaranteed to remain up-to-date as the source ontologies evolve (Hastings et al., 2015). Several other projects have recognized the need for a harmonized terminology, and a technical report on this issue was recently published by the European Joint Research Center of the European Commisssion. Further refinement of the ontology developed in eNANOMAPPER is ongoing in other EU-funded projects, for instance the H2020 project, NanoReg2.

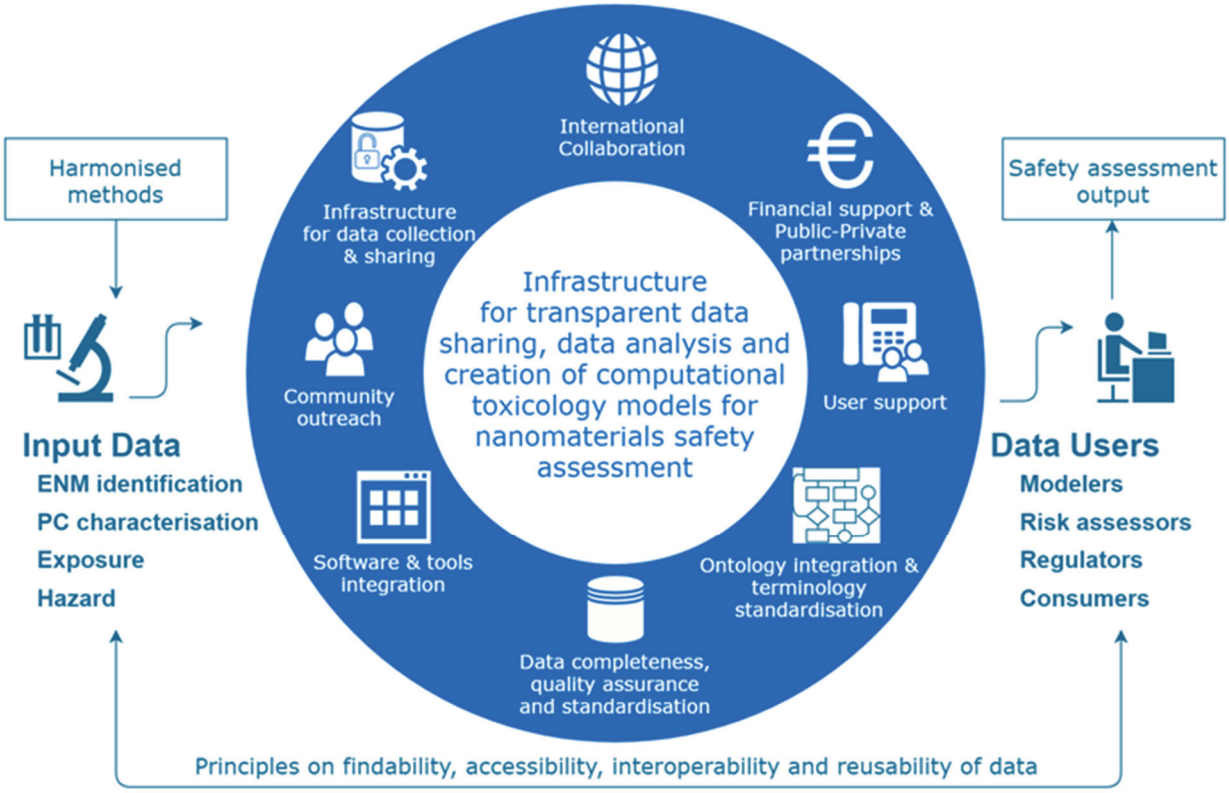


Figure 15. Proposal for a harmonized knowledge infrastructure supporting environment, health and safety assessment of ENMs taking into account the FAIR data principles of findability, accessibility, interoperability, and reusability of data. Fadeel et al., 2018.

The solutions described above efficiently support curation of nanosafety data into standardized data templates, proper annotation with a common ontology and storage in an electronic, easily accessible and widely linked database (Powers et al., 2015). Implementation of well-defined curation workflows plays a role in maximizing the effectiveness of data gathering and sustainability of common resources, and in addition provides information about standardization possibilities, common bottlenecks and leverage points that benefit the community as a whole (Figure 15). Furthermore, to pursue the optimal usage of this data, such workflows should consider the FAIR data principles of findability, accessibility, interoperability and reusability of data and the algorithms, tools and workflows that operate on it. Curated nanosafety data can be assumed to be sufficiently complete and of sufficient quality to serve its intended purpose of answering particular scientific or regulatory questions, and the curation process can include assessment and labelling of the level of completeness and quality. To date, however, assessing data

completeness and quality has proven particularly difficult due to the lack of harmonized and standardized storage of nanosafety data. A few initiatives have nevertheless been developed and are based on minimum information checklists and toxicology data quality schemes (Marchese Robinson et al., 2015). For example, the Nanomaterials Registry Initiative at the US National Institutes of Health has developed a 'minimal information about nanomaterials' (MIAN) in order to be able to establish evaluation and similarity measures for data curated into the Registry (Ostraat et al., 2013). Based on this, a compliance-level score is calculated that serves as a quality and quantity label of mainly the physicochemical characterization data performed for a specific ENM. Further suggestions on how to evaluate completeness and quality of curated nanomaterial data include recommendations on terminology standardization, and computational tools to support evaluation of completeness and quality, as well as the role of specific organizations and scientific communities in advancing the manner in which the completeness and quality of curated nanomaterial data are evaluated (Marchese Robinson et al., 2016). The EU-funded H2020 project CALIBRATE seeks to implement these recommendations and is working towards high-quality data supported nano risk governance and safe development of nano-enabled products. Overall, efforts are being made to develop a harmonized knowledge infrastructure in support of nanosafety research and nano risk governance.

References

Ashburner M, et al. Gene ontology: tool for the unification of biology. The gene ontology consortium. Nat Genet. 2000(1):25-9.

Brazma A, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001;29(4):365-71.

Fadeel B, Farcal L, Hardy B, Vázquez-Campos S, Hristozov D, Marcomini A, Lynch I, Valsami-Jones E, Alenius H, Savolainen K. Advanced tools for the safety assessment of nanomaterials. Nat Nanotechnol. 2018;13(7):537-543.

Hastings J, Jeliazkova N, Owen G, Tsiliki G, Munteanu CR, Steinbeck C, Willighagen E. eNanoMapper: harnessing ontologies to enable data integration for nanomaterial risk assessment. J Biomed Semantics. 2015;6:10.

Hendren CO, Powers CM, Hoover MD, Harper SL. The Nanomaterial Data Curation Initiative: a collaborative approach to assessing, evaluating, and advancing the state of the field. Beilstein J Nanotechnol. 2015;6:1752-62.

Jeliazkova N, et al. The eNanoMapper database for nanomaterial safety information. Beilstein J Nanotechnol. 2015;6:1609-34.

Marchese Robinson RL, et al. How should the completeness and quality of curated nanomaterial data be evaluated? Nanoscale. 2016;8(19):9919-43.

Ostraat ML, Mills KC, Guzan KA, Murry D. The Nanomaterial Registry: facilitating the sharing and analysis of data in the diverse nanomaterial community. Int J Nanomedicine. 2013;8 Suppl 1:7-13.

Powers CM, Mills KA, Morris SA, Klaessig F, Gaheen S, Lewinski N, Ogilvie Hendren C. Nanocuration workflows: establishing best practices for identifying, inputting, and sharing data to inform decisions on nanomaterials. Beilstein J Nanotechnol. 2015;6:1860-71.

Sansone SA, et al. Toward interoperable bioscience data. Nat Genet. 2012;44(2):121-6.

Thomas DG, Gaheen S, Harper SL, Fritts M, Klaessig F, Hahn-Dantona E, Paik D, Pan S, Stafford GA, Freund ET, Klemm JD, Baker NA. ISA-TAB-Nano: a specification for sharing nanomaterial research data in spreadsheet-based format. BMC Biotechnol. 2013;13:2.

Thomas DG, Pappu RV, Baker NA. NanoParticle Ontology for cancer nanotechnology research. J Biomed Inform. 2011;44(1):59-74.

Wilkinson MD, et al. The FAIR guiding principles for scientific data management and stewardship. Sci Data. 2016;3:160018.

6. Risk Assessment

6.1. Limitations of Traditional Risk Assessment Approaches

For nanomaterials just as for other chemical compounds the risk depends on both the intrinsic *hazard* of the compound as well as the *exposur*e. In a traditional risk assessment of a chemical substance the hazard is first identified, e.g., by using OECD accepted methods. The nature of the hazard can be acute (such as skin corrosion) or chronic (such as carcinogenicity). Generally, the available studies assessing local toxicity indicate a low eye or skin-irritating potential of a broad spectrum of ENMs (Landsiedel et al., 2017). Hazard to various organs as well as genotoxicity and carcinogenicity of ENMs are discussed in other chapters of the current report. For assessing the health risks of chemicals or nanomaterials, it is of importance to understand at which doses the effects occur in order to derive a no-observed-adverse-effect-level (NOAEL). This is the highest exposure level at which no statistically or biologically significant increases are seen in the frequency or severity of adverse effects. Such a value can then be used as a starting point or point-of-departure (POD) to derive a reference dose that can be used in health risk assessment. This is often done by dividing the POD value with assessment factors to take into account the variability between animals and humans as well as susceptibility within a population. Following exposure assessment, the exposure levels can be compared to the reference dose and if the reference dose is not exceeded, the risk is considered acceptable (risk characterization). The risk assessment can, for instance, lead to the calculation of a tolerable daily intake (TDI) or occupational limit value.

Traditionally, chemical risk assessments have been made on a case-by-case basis and this has been extremely time consuming. Therefore, very little information regarding toxicological properties is available for most chemicals. In 2007, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation entered into force in the European Union. REACH requires that a substance is registered before being placed into the market. The information needed in the registration dossier varies according to the volume of production (tonnage level) of the substance. If the substance exceeds 10 tons/year or is classified as having hazardous properties (e.g., carcinogenicity, acute toxicity in aquatic species), a chemical safety assessment (CSA) needs to be performed. The CSA should contain the three main steps: hazard assessment exposure assessment, and risk characterization. REACH deals with substances in whatever size, shape or physical state they come, and therefore, ENMs are also covered by the definition of 'substance' under REACH. Nanomaterials are, however, not mentioned in the legal text just

as it does not explicitly refer to fibres. In 2017, ECHA published a REACH 'guidance for nanomaterials' in order to help registrants to prepare dossiers that cover nanoforms. To further clarify the need for information on the nanoforms of substances, the Member States recently (April 2018) agreed to amend several annexes to REACH with specific requirements for nanomaterials (Clausen and Hansen, 2018). The requirements include information on basic characteristics, use, how to handle the nanomaterials safely, what risks they potentially pose to health and the environment and how these risks can be adequately controlled. After scrutiny by the European Parliament and Council the new amendments can be adopted by the European Commission and enter into force in 2020. Application of the full REACH information requirements for every single variant of a given nanomaterial taking into account different sizes, shapes or surface properties would lead to an enormous amount of testing and would stand in contradiction to the requirement to replace, reduce, and refine animal testing (the '3Rs principle'). In addition to the vast number of possible different ENMs, the risk assessment situation is even more complicated when considering the life-cycle of a product containing ENMs. For example, the risk presented by carbon nanotubes (CNTs) in the immediate production, for instance, may be considerably different from the risk presented by processed or purified nanotubes. Likewise, once the CNTs have been incorporated into a product, the exposure potential as well as the physicochemical nature of material being released is profoundly different from that of the starting material, just as the possible exposure changes when the product is disposed or recycled (Maynard, 2016). Due to this complexity, much attention has been placed on exposure potential as a first estimate of potential risk, leading to a focus on workplace exposure. This has also led to suggestions for use of so-called control banding tools in order to manage the risk in the absence of firm toxicological and exposure information (Brouwer, 2012). We shall discus such tools as well as grouping and other risk assessment approaches below.

6.2. Existing Risk Assessment Approaches for Nanomaterials

The number of different ENMs already used in society constitutes a problem related to prioritization and approaches to use for risk assessment. Indeed, it is virtually impossible to evaluate each nanoparticle type in a case-by-case approach and the need for grouping and read across approaches is evident. As previously mentioned, the challenges with risk assessment of ENMs related, for instance, to a lack of data has led to various proposals for prioritization of ENMs. One such approach that

may be helpful to assess risks in the work environment is “Stoffenmanager Nano” (https://nano.stoffenmanager.nl). This ‘work-in-process’ online tool combines available hazard information with an inhalation exposure estimate. Furthermore, when risks emerge, measures to control these can be investigated by using the tool. ENMs that fulfill certain critera – eg., being less than 100 nm (also agglomerates) and not water soluble can be handled. Several EU-funded projects, including FP7-SUN and FP7-GUIDENANO, have also developed tools to support risk assessment and risk management (Fadeel et al., 2018). Furthermore, a wide range of different grouping or categorization concepts have been developed, for instance within the frame of the EU-funded NanoReg2 project. One of the most well-known approaches is the so-called ‘decision-making framework for the grouping and testing of nanomaterials’ (aka DF4nanoGrouping) that has been elaborated by the ‘Nano-Task Force’ of ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) (Arts et al., 2015; *idem*, 2016). This strategy consists of three tiers that will assign ENMs to four main groups. These main groups are: (1) soluble nanomaterials; (2) biopersistent high-aspect ratio (HAR) nanomaterials; (3) passive nanomaterials including ‘granular biodurable particles without specific toxicity’, GBPs; and (4) ‘active’ nanomaterials. The different tiers used to determine the group are: Tier 1: initial characterization of the nanoparticles in terms of water solubility, particle morphology, chemical composition; Tier 2: assessment of additional properties including dissolution in biological media, surface reactivity, particle dispersibility; and Tier 3: confirmation of Tier 1 and 2 groups is confirmed or corrected by using data from short-term *in vivo* studies, such as rat STIS or rat intratracheal instillation studies. DF4nanoGrouping allows for subgrouping of ENMs in group 4 (active nanomaterials) by their pattern of biodistribution and thus distinguishes between ENMs that only become available in the primary organ (often the lungs), ENMs that also are found in the mononuclear phagocytic system, and ENMs that also become systemically available. A strategy for risk assessment of nanomaterials was recently developed in the FP7-NANOREG project (Dekkers et al., 2016). This strategy builds on work in previous projects such as ITS-NANO (Stone et al., 2014), MARINA (Oomen et al., 2015), and several other recent projects (Dekkers et al., 2016).

Although it is still not entirely clear whether any nano-specific hazards exist or whether the toxicity of ENMs is merely a gradual magnification of the intrinsic hazard posed by decreasing the size (Donaldson and Poland, 2013), the NANOREG approach is focused on nano-specific issues not only in relation to hazard, but also the exposure assessment and kinetic

behaviour. The nanospecific behaviour is considered to be especially relevant for: (a) exposure (deposition and agglomeration), (b) absorption and distribution (transport across biological barriers like gut epithelium, blood-brain barrier, or skin), (c) accumulation, and (d) toxic potency (dose-response relationships) (Dekkers et al., 2016). The approach is focused on six main elements namely: exposure potential, dissolution, transformation, accumulation, genotoxicity, and immunotoxicity (Figure 16).

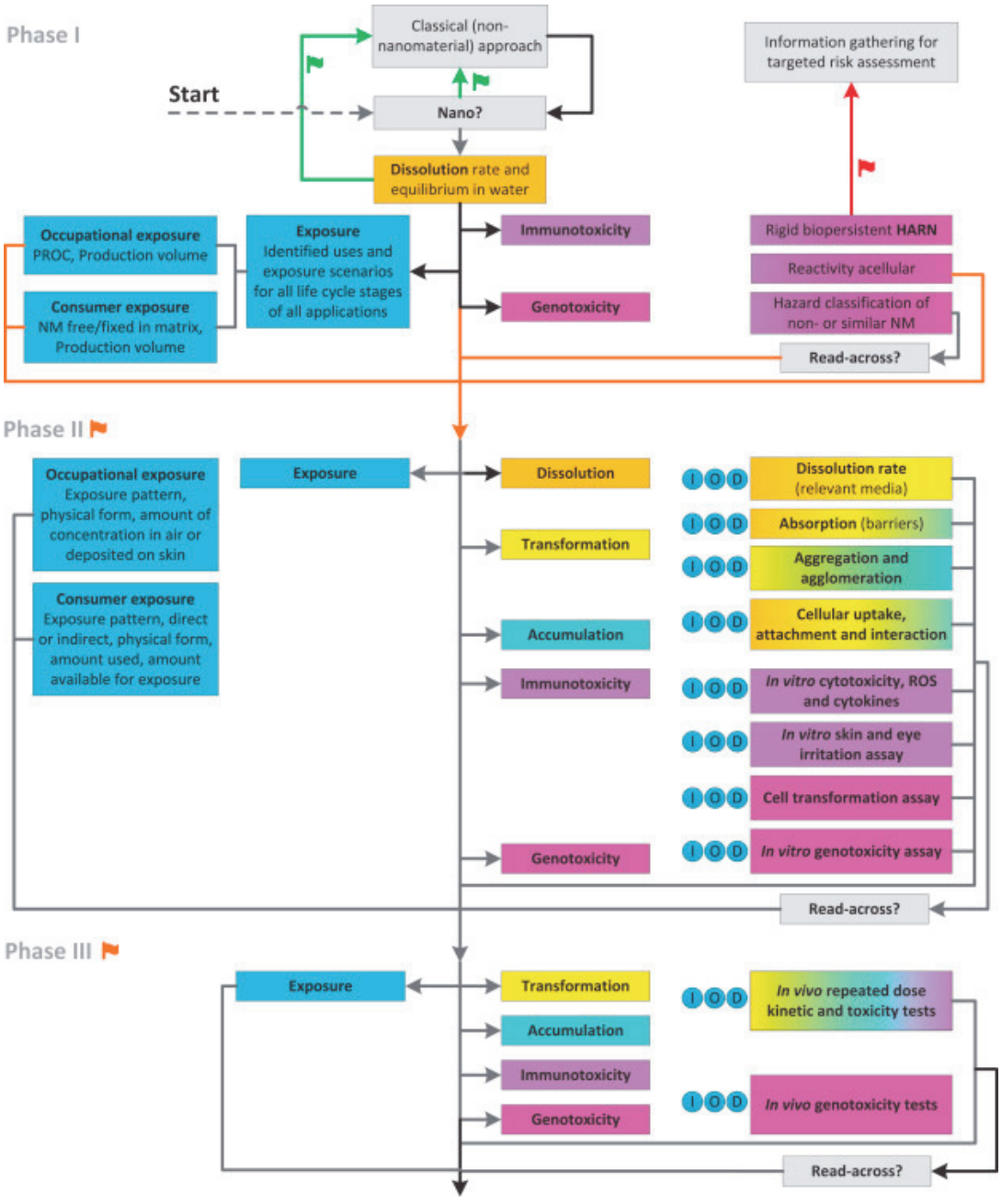


Figure 16. NANOREG proposal for a nanospecific risk assessment. Dekkers et al. (2016).

The first phase of the NANOREG approach deals with screening and prioritization and from this phase one should be able to obtain a first idea on the possible adverse health effect of a specific material. This is done by identifying: (a) materials that have the highest potential to be hazardous (flagged red), (b) materials for which the classical (non-nanomaterial) risk assessment approach can be performed (flagged green), and (c) materials that need further evaluation (flagged orange). One early element to consider is the dissolution rate. The main reason is that if the nanoparticle quickly dissolves, then it could be possible to use existing data on the soluble form of the substance (no nano-specific risk assessment needed). Here, a cut-off value on dissolution rate is needed in order to distinguish the nanoparticles which almost immediately dissolve. The likelihood for exposure is also taken into account in the first phase, as well as whether it is a HARN (immediately flagged red) and the acellular reactivity. It is likely that the orange group will be the largest group and a ranking of these will be needed in order to understand the potential to cause harmful effects. The orange ones are considered in phase II. In the second phase those nanoparticles flagged orange will be further considered regarding the main exposure routes and *in vitro* assays for absorption, cytotoxicity, immunotoxicity, genotoxicity, etc. The dissolution rate in relevant media is also considered since this can have a great impact on the exposure potential and behavior in humans, including translocation to secondary organs (Dekkers et al., 2016). In contrast to the output of phase I, the information obtained in this phase is not used for ranking of different nanomaterial-applications but gives direction regarding the information needed in phase III. In the third phase, the information provided in phase II will be decisive for how to proceed in terms of *in vivo* studies. For example, positive results of *in vitro* cell transformation assays and *in vivo* genotoxicity in combination with observed systemic availability and toxicity (such as inflammatory effects) may trigger long-term, repeated exposure studies.

Concluding remarks

Risk assessment of nanomaterials is challenging. Efficient protection in a work environment can be achieved by avoiding exposure (‘prevention by design’). Different tools and approaches for tiered-testing and grouping have been developed in recent years. Case studies need to be performed to better understand the usefulness of these approaches. Nanoparticles that immediately dissolve are unlikely to pose nano-specific risks. Certain properties of nanoparticles are, however, clearly considered to be of

concern, such as high surface area and reactivity. Furthermore, biopersistent, high-aspect ratio materials are considered to pose a risk. Occupational exposure limits (OELs) for ENMs should also be developed.

References

Arts, J. H., Hadi, M., Irfan, M. A., Keene, A. M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Petry, T., Sauer, U. G., et al. (2015). A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regulatory toxicology and pharmacology : RTP 71(2 Suppl), S1-27.

Arts, J. H., Irfan, M. A., Keene, A. M., Kreiling, R., Lyon, D., Maier, M., Michel, K., Neubauer, N., Petry, T., Sauer, U. G., et al. (2016). Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regulatory toxicology and pharmacology : RTP 76, 234-61.

Brouwer, D. H. (2012). Control banding approaches for nanomaterials. The Annals of occupational hygiene 56(5), 506-14.

Clausen LPW, Hansen SF. The ten decrees of nanomaterials regulations. Nat Nanotechnol. 2018;13(9):766-768.

Dekkers, S., Oomen, A. G., Bleeker, E. A., Vandebriel, R. J., Micheletti, C., Cabellos, J., Janer, G., Fuentes, N., Vazquez-Campos, S., Borges, T., et al. (2016). Towards a nanospecific approach for risk assessment. Regulatory toxicology and pharmacology. 80:46-59.

Donaldson, K., and Poland, C. A. (2013). Nanotoxicity: challenging the myth of nano-specific toxicity. Current opinion in biotechnology 24(4), 724-34.

ECHA, JRC, and RIVM (2016). Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance. Elements to consider. ISBN: 978-92-9247-810-0.

Fadeel B, Farcal L, Hardy B, Vázquez-Campos S, Hristozov D, Marcomini A, Lynch I, Valsami-Jones E, Alenius H, Savolainen K. Advanced tools for the safety assessment of nanomaterials. Nat Nanotechnol. 2018;13(7):537-543.

Hansen, S. F., Larsen, B. H., Olsen, S. I., and Baun, A. (2007). Categorization framework to aid hazard identification of nanomaterials. Nanotoxicology, 243-250.

Kuempel, E. D., Castranova, V., Geraci, C. L., and Schulte, P. A. (2012). Development of risk-based nanomaterial groups for occupational exposure control. Journal of nanoparticle research: an interdisciplinary forum for nanoscale science and technology 14, 1029.

Landsiedel, R., Sauer, U.G., de Jong, W. Risk Assessment and Risk Management. In: *Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology, and Impact on Human Health*. Eds: Fadeel B, Pietroiusti A, Shvedova A. Elsevier. 2nd Edition. (2017).

Lynch, I., Weiss, C., and Valsami-Jones, E. (2014). A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs. Nano Today 9(3), 266-270.

Maynard (2016). The Challenge of Nanomaterial Risk Assessment. In: *Assessing Nanoparticle Risks to Human Health*. Ed. G. Ramachandran. Elsevier. Second Edition.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., and Zhang, H. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Accounts of chemical research 46(3), 607-21.

Oomen, A. G., Bleeker, E. A., Bos, P. M., van Broekhuizen, F., Gottardo, S., Groenewold, M., Hristozov, D., Hund-Rinke, K., Irfan, M. A., Marcomini, A., et al. (2015). Grouping and Read-Across Approaches for Risk Assessment of Nanomaterials. International journal of environmental research and public health 12(10), 13415-34.

Sellers, K., Deleebeeck, N. M. E., Messiean, M., Jackson, M., Bleeker, E. A. J., Sijm, D. T., and van Broekhuizen, F. A. (2015). Grouping nanomaterials : A strategy towards grouping and read-across. RIVM Report 2015-0061. 1-232.

Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., et al. (2014). ITS-NANO--prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy. Particle and fibre toxicology 11, 9.

7. Conclusions and Future Research Needs

In 2006, Maynard et al. presented five grand challenges in the hope that this would galvanize research that is relevant to safety of nanotechnologies (Maynard et al., 2006). The challenges addressed the need to: a) develop instruments to assess exposure to nanomaterials in air and water; b) develop and validate methods to evaluate the toxicity of nanomaterials; c) develop models for predicting the potential impact of nanomaterials on the environment and human health; d) develop systems for evaluating the health and environmental impact of nanomaterials over their entire life; and e) develop strategic programmes that enable relevant risk-focused research. There has been considerable progress with respect to strategic programmes on nanosafety both in the EU (www.nanosafetycluster.eu) and in the United States (www.nano.gov). In Sweden, a national platform was launched in 2016 with the aim to support and strengthen communication between different stakeholders (www.swenanosafe.se) and there are also some efforts to coordinate nanosafety research at the national level (www.mistraenvironmentalnanosafety.org), although there is no national strategy for nanosafety, and strategic research investments in this area remain limited. Furthermore, considerable research efforts have been directed towards evaluating the toxicity of ENMs, but the relevance of these studies for risk assessment has been called into question (Krug, 2014), and some have argued that mechanistic understanding remains limited despite much research (Valsami-Jones and Lynch, 2015). This may be true if we mean a predictable pattern of toxicity that can explain the effects of all different types of ENMs. However, for some specific classes of ENMs, we now have a good understanding of the potential risks associated, for instance, with the fiber-like properties of these materials (Kane and Hurt, 2008). Similarly, for nanoparticles such as nano-Ag that undergo rapid dissolution, the underlying principle of toxicity is well understood and not nano-specific (Hansen and Baun, 2012). Furthermore, it is now widely accepted that one must take care to characterize the materials that are subjected to testing, not only the pristine particles, but also particles released from nano-enabled products under realistic conditions. The test methods also need to be validated and a proposal was recently made for minimum reporting requirements for any publication dealing with biological interactions of ENMs (Faria et al., 2018). On the other hand, research has been lagging behind in terms of exposure measurements (Fadeel and Savolainen, 2013) as well as the predictive modeling of the impact of nanomaterials on health and the environment (Maynard and Aitken, 2016).

“Funding agencies should ensure that nanotechnology development takes safety issues into account”

The present report has provided a summary of the state-of-the-art of nanotoxicology, with emphasis on hazard assessment of ENMs for human health. From this survey of the literature, and on the basis of recent and ongoing research activities at the Institute of Environmental Medicine (IMM), we can conclude that considerable progress is being made, for instance, with respect to the implementation of advanced *in vitro* models for toxicity testing of nanomaterials and *in silico* modeling to evaluate the biodistribution of nanomaterials as well as systems biology approaches with which to dissect the mechanisms of action of nanomaterials (Fadeel et al., 2018). Nevertheless, we suggest that further, concerted research efforts are needed at the national level to enable safe handling of nanomaterials. We summarize the research challenges below under four main headings: material characterization, exposure assessment, hazard assessment, and risk assessment. These national research efforts should, of course, be aligned with ongoing international activities. Furthermore, the national platform for safe handling of ENMs, SweNanoSafe (www.swenanosafe.se) should continue to support knowledge exchange between relevant stakeholders. In addition, the elaboration of a national strategic plan for nanotechnology research and innovation with safety assessment at every step of the innovation process is also seen as an urgent, near-future goal. Funding agencies should ensure that nanotechnology research will take safety issues into account (i.e., responsible research and innovation) at every stage of development.

Material characterization:

- develop minimal characterization requirements taking into account the material-intrinsic properties as well as the acquired biological identity or bio-corona
- monitor the transformation of ENMs in living systems and the environment
- conduct real-time material characterization linked to hazard and exposure assessment
- develop libraries of ENMs to support structure-activity relationship studies and establish reference nanomaterials for cross-laboratory comparisons

Exposure assessment:

- define relevant exposure metrics in relation to biological/medical effects of ENMs
- develop laboratory and computer simulations to model exposure and life-cycle analysis
- identify cohorts of exposed workers, set up health surveillance, investigate exposure conditions, analyze markers of exposure and effects
- investigate chronic effects of exposure to ENMs, eg. cancer, cardiovascular and pulmonary disease in exposed populations, eg., workers

Hazard assessment:

- establish validated test methods with improved *in vitro* to *in vivo* predictivity, and support focused, integrated testing strategies and safe-by-design approaches
- advanced *in vitro* test methods to replace animal testing including methods for assessment of low-dose, long-term (chronic) effects of ENM
- develop tests that address the impact on vulnerable populations including young and old
- develop HTS assays based on validated mechanisms of action for ENMs
- explore systems biology and modeling approaches to unearth structure-activity relationships and determine modes action of ENMs

Risk assessment:

- data storage solutions and open access requirements to make research data available and useful for modeling purposes and risk assessment
- specific funding for research that produces results relevant for risk assessment
- further studies aimed at understanding the relation between external exposure and target dose to enable *in vitro* to *in vivo* prediction of toxicity
- development and implementation of occupational exposure limit (OEL) values
- development of adverse outcome pathways (AOPs) and grouping approaches

References

Fadeel B, Farcal L, Hardy B, Vázquez-Campos S, Hristozov D, Marcomini A, Lynch I, Valsami-Jones E, Alenius H, Savolainen K. Advanced tools for the safety assessment of nanomaterials. Nat Nanotechnol. 2018;13(7):537-543.

Fadeel B, Savolainen K. Broaden the discussion. Nat Nanotechnol. 2013;8(2):71.

Faria M, Björnmalm M, Thurecht KJ, Kent SJ, Parton RG, et al. Minimum information reporting in bio-nano experimental literature. Nat Nanotechnol. 2018;13(9):777-785.

Hansen SF, Baun A. When enough is enough. Nat Nanotechnol. 2012;7(7):409-11.

Kane AB, Hurt RH. Nanotoxicology: the asbestos analogy revisited. Nat Nanotechnol. 2008;3(7):378-9.

Krug HF. Nanosafety research--are we on the right track? Angew Chem Int Ed Engl. 2014;53(46):12304-19.

Maynard AD, Aitken RJ. Safe handling of nanotechnology ten years on. Nat Nanotechnol. 2016;11(12):998-1000.

Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, et al. Safe handling of nanotechnology. Nature. 2006;444(7117):267-9.

Valsami-Jones E, Lynch I. Nanosafety. How safe are nanomaterials? Science. 2015;350(6259):388-9.

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