NanoImpact 28 (2022) 100416

Contents lists available at ScienceDirect

NanoImpact

journal homepage: www.elsevier.com/locate/nanoimpact

Review article

Analytical and toxicological aspects of nanomaterials in different product groups: Challenges and opportunities

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ARTICLE INFO

Editor: Dr. Phil Demokritou

Keywords:
Nanomaterials
Release
Human Nanotoxicology
Advanced analytics
Risk assessment
NM-containing products

ABSTRACT

The widespread integration of engineered nanomaterials into consumer and industrial products creates new challenges and requires innovative approaches in terms of design, testing, reliability, and safety of nanotechnology. The aim of this review article is to give an overview of different product groups in which nanomaterials are present and outline their safety aspects for consumers. Here, release of nanomaterials and related analytical challenges and solutions as well as toxicological considerations, such as dose-metrics, are discussed. Additionally, the utilization of engineered nanomaterials as pharmaceuticals or nutraceuticals to deliver and release cargo molecules is covered. Furthermore, critical pathways for human exposure to nanomaterials, namely inhalation and ingestion, are discussed in the context of risk assessment. Analysis of NMs in food, innovative medicine or food contact materials is discussed. Specific focus is on the presence and release of nanomaterials, including whether nanomaterials can migrate from polymer nanocomposites used in food contact materials. With regard to the toxicology and toxicokinetics of nanomaterials, aspects of dose metrics of inhalation toxicity as well as

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https://doi.org/10.1016/j.impact.2022.100416
Received 14 January 2022; Received in revised form 15 July 2022; Accepted 14 August 2022
Available online 20 August 2022

ingestion toxicology and comparison between *in vitro* and *in vivo* conclusions are considered. The definition of dose descriptors to be applied in toxicological testing is emphasized. In relation to potential exposure from different products, opportunities arising from the use of advanced analytical techniques in more unique scenarios such as release of nanomaterials from medical devices such as orthopedic implants are addressed. Alongside higher product performance and complexity, further challenges regarding material characterization and safety, as well as acceptance by the general public are expected.

1. Introduction

Nanotechnology has become integrated in numerous areas of daily life. The application of engineered nanomaterials (ENM) in almost all areas of human activity, for example in food and medicine, water, or in consumer products, creates new challenges. ENMs are deliberately designed and prepared materials in the nanoscale. In addition to ENMs there are those nanomaterials (NM) not derived from an engineered process, but of natural occurrence or incidental origin. Therefore, innovative approaches to design, testing, reliability, and safety are required (Oomen et al., 2018; Pöttler et al., 2019; Morrell et al., 2019; Kraegeloh et al., 2018; Correia et al., 2019a). Recent innovations and new scientific concepts are discussed in relation to novel nano-enabled products, utilizing the benefit of the unique size-dependent properties of ENMs (Wohlleben et al., 2017). Furthermore, the development of reliable detection and characterization methods for NMs (engineered and incidental) present in and released from for example food, food contact materials (FCM) and also more specifically from e.g. implanted medical device or spray products, which may potentially result in direct human exposure, are of particular importance (Fig. 1). Here, techniques such as inductively coupled plasma-mass spectrometry in single particle mode (spICP-MS) can give a valuable understanding of NMs (Mozhayeva and Engelhard, 2020; Laux et al., 2018), especially for risk assessment scenarios that involve complex matrices such as the presence of NMs in various foods, and for confirming nano-content labelling. Another scenario is the release of wear NMs from medical devices such as metal implants (Schoon et al., 2017a), resulting in incidental NMs. Current advances in nano-sized surface modifications will potentially lead to even higher complexity regarding risk-benefit evaluation of NMs released from, e.g., metallic biomedical materials (Makvandi et al., 2020).

For manufactured NMs, the OECD Working Party on Manufactured Nanomaterials (WPMN) concluded that, as for general chemicals, the risk assessment of NMs relies on the determination of possible exposure and hazards through dose-response analysis (OECD, 2013). Human health hazard assessment of chemicals, including NMs, involves a comprehensive analysis of toxicological studies. The OECD's test guideline programme currently fine-tunes the applicability of its test guidelines to NMs as well as develops new ones. The first test guidelines that were fine-tuned were Test No. 412: Subacute Inhalation Toxicity: 28-Day Study and Test No. 413: Subchronic Inhalation Toxicity: 90-Day Study as well as the associated Guidance Document 39 on Acute Inhalation Toxicity Testing (OECD, 2018a; OECD, 2018b; OECD, 2018c). For medicinal products, the European Medicines Agency (EMA) also requires clinical studies. *In vitro* and in silico data are increasingly used in toxicological testing. Either before animal studies are performed or in addition to them. Furthermore, epidemiological studies may be relevant. The relevant studies to be conducted include different exposure routes such as inhalation, oral, and dermal exposure; for clinical studies, intravenous exposure is also relevant. Historically, exposure concentration (i.e., amount of substance per volume of liquid or air) was used for dose-response relations reported in these types of studies. However, from a biological point of view, the dose delivered to cells and/or organisms is unambiguously governing the toxicological response. This dose cannot be determined directly from the reported exposure concentration/ dose administered to the cell culture or the organism. Therefore, this article discusses the relevance of the tissue-delivered dose in NM risk assessment (Schmid and Cassee, 2017).

Toxicological research for assessing hazards of NMs is evolving towards mechanistic elucidation of toxicological pathways (mode of action) and consideration of more relevant dose metrics than mass, such as volume and surface area (Laux et al., 2018; Schmid and Stoeger, 2016). Unfortunately, mass is the most commonly used dose parameter, although it is not sufficiently informative in toxicological studies of chemicals and poorly or insoluble NMs. In this context, hazard classification is often more potent if the functional entity of interaction is

Product groups	Analytical challenges	Toxicological considerations	Limitations/ Knowledge gaps and Future needs
Food	Analysis in matrices	Oral toxicity	• Differentiation between sources • Need for validation and reference materials
Food contact materials	Release and possible migration	Oral toxicity	Case-by-case risk assessment
Spray applications	• Aerosol dynamics • Chemical reactions	Inhalation toxicity	Identification of most appropriate dose metrics
Medical devices	Detection and quantification of long-term release in humans	Focus on systemic effects	Need for toxicokinetic studies
Pharmaceuticals and Nutraceuticals	Controlled release	Mode of action	Improvement of bioavailability and therapeutic success

Fig. 1. Overview of nanomaterial-containing product groups of special concern and their respective issues in regard to characterization and analysis, toxicology, as well as knowledge gaps/future research needs. *Created with BioRender.com.*

considered as dose metric, namely the number of molecules (molarity) and surface area (Schmid and Cassee, 2017; Krug, 2014; Sieg et al., 2017).

Generally, bioresorbable ENMs, which are designed to degrade within the body after performing their function, are regarded as less critical. This is supported by advantages resulting from their use and temporary presence in the human body. Bioresorbable ENMs show interesting benefits as nano-sized delivery system such as overcoming biological barriers more effective than conventional medicine (Bourganis et al., 2018; Ahmadi et al., 2020), enabling specific targeting of organoids and cells, as well as protection of sensitive cargo molecules in food and medicine.

Ingestion and inhalation are considered the most crucial routes of exposure to NMs, apart from injection or in human generated NMs, as such rreleased from medical implants inside a human body, which are immediately systemically available. Post-inhalation effects include lung overload due to impaired lung clearance and subsequent inflammation, possibly leading to cancer development. These effects were initially based on volumetric considerations and have also been related to the pool-size of bronchoalveolar lavage (BAL)-cells (Li and Pauluhn, 2018). Furthermore, there are scientific arguments that particle surface area is a suitable metric to describe toxicological effects of NMs. In general, the generation of toxicokinetic data is considered crucial for the mechanistic explanation of toxic effects such as lung tumours, and the evaluation of dose-response relationships. Contrary to the assumptions of Morrow et al., no lung burden threshold was identified for lung clearance impairment in a recent chronic *in vivo* study of the carcinogenicity of nano-CeO_2 in rats (Tentschert et al., 2020; Morrow, 1988).

The objective of this review article is to provide insight into several NM-containing product groups of particular interest. The groups were selected due to the lack of data and the clear need for research in terms of analysis, exposure or toxicology (Fig. 1), thus overall safety. Toxic effects are highly dependent on the route of exposure, which affects absorption and internal exposure levels. Relevant consumer exposure to NM can occur through inhalation, oral ingestion and dermal absorption. Relevant products groups for exposure are food, food contact materials, medical devices, spray applications (aerosolized NM), and nutraceuticals, as well as pharmaceuticals such as nanomedicines. In addition, more specific scenarios such as the release of NMs from medical devices, e.g., metallic biomedical materials, will be highlighted and the use of NMs for beneficial purposes, such as the delivery and release of cargo molecules, will be addressed. Product groups are discussed in terms of analytical and toxicological challenges, solutions, and limitations, with emphasis on risk assessment and identification of future research needs.

2. Nanomaterials in selected product groups of special concern for consumers

Different product groups can be prioritized in terms of relevance for consumers by either having increased exposure or unwanted health effects. This chapter takes a closer look at certain product groups identified as relevant and gives examples of beneficial application in daily life and medicine.

2.1. Food

Food is a major source of consumer exposure to inorganic NMs via the oral route (Fig. 2, A). Hence, food additives such as SiO_2 (E551), TiO_2 (E171), iron oxides (E172) or Al_2O_3 (E173) which are upon the most used additives are of particular relevance (Cao et al., 2019; Sieg et al., 2018).

2.1.1. Presence and analysis of inorganic nanomaterials in food

In the context of food labelling, risk assessment and the development

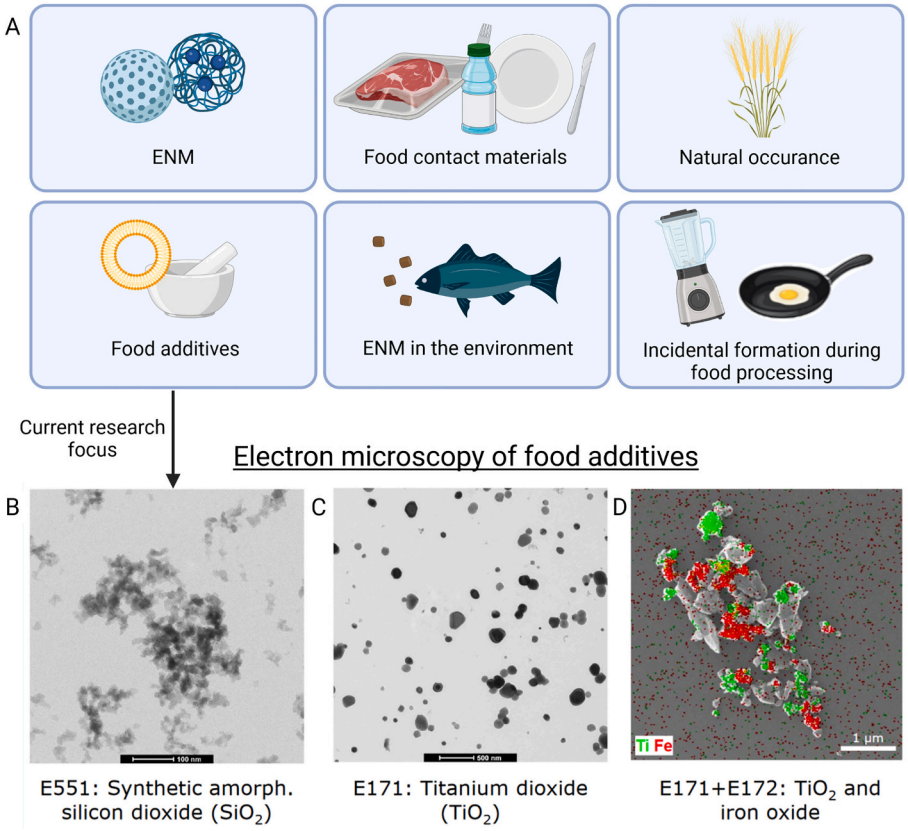

Fig. 2. (A) Potential sources of NMs in food; (B-D) Electron microscopy images of common food additives potentially containing a fraction of material at the nanoscale. Modified with permission from (Correia et al., 2019b). Copyright 2022 Elsevier. *Created with BioRender.com.*

of novel foods, reliable detection and characterization methods for ENMs/NMs in food are needed. Therefore, in particular spICP-MS has become a frequently used technique as a highly sensitive screening method for inorganic NMs. Examples of its application to food matrices include the detection of lead NMs in game meat and aluminum-containing NMs in noodles (Kollander et al., 2017; Loeschner et al., 2018). However, care has to be taken with regard to false-positive-results and the obtained quantitative information in terms of particle size distribution (PSD) and number / mass concentration. The relatively easy implementation of spICP-MS in state-of-the-art ICP-MS instruments, despite the size limitation of about 20 nm for certain elements, makes it a promising technique for routine analysis of NMs (Rosenkranz et al., 2020; Venkatesan et al., 2018). Standard ICP-MS instruments, on the other hand, are mostly used for metal analysis and speciation. Another often applied technique is asymmetric flow field flow fractionation (AF4) hyphenated with ICP-MS, examples include the analysis of Al_2O_3 and TiO_2 particles in toothpaste and of nanoplastics spiked to fish (Correia et al., 2018; Correia and Loeschner, 2018). AF4-ICP-MS can be a very helpful technique for characterizing complex samples and for obtaining reliable mass-based PSDs, but it is not an ideal method for determining the number-based PSD. In order to acquire accurate data, the AF4 separation method and settings must be optimized for each new sample matrix and NM combination. Electron microscopy is always required as confirmative technique, as it is the only technique that provides information on particle shape and distinguishes individual particles from particle aggregates or agglomerates. Each of the analytical techniques provides only limited information, e.g., size range, limit of detection for mass/number concentration, or evaluation of the data relies on the assumption of spherical particles. Therefore, a combination of several techniques is usually required. Sample preparation can be identified as a crucial step, especially in the case of solid / semi-solid matrices where simple dilution is not sufficient. As spICP-MS analysis is not as sensitive to possible remaining matrix residues as AF4, complete digestion of the matrix is usually not required. The main challenge is to minimize changes, such as dissolution or aggregation, of the NMs during sample preparation. For the majority of food samples, enzymatic digestion can be identified as the most suitable sample preparation method.

2.1.2. Limitations and future needs for risk assessment of NMs in food

In general, only few validated methods for identifying NMs in food exists and most methods are typically only tested by one laboratory. The developed methods are often specific to certain NM / food matrix combinations and sometimes even NM characteristics such as surface properties. The trueness of results, especially size, cannot be determined due to the lack of certified reference materials, which is particularly challenging during method development. This emphasizes a growing need for validation, standardization, and implementation of testing methods and tools needed for a comprehensive regulatory risk assessment to ensure nano-safety.

2.1.3. Health effects by NM in food

Inorganic NMs in food and drinks are typically ingested via the oral route. The stability and bioavailability of NMs in the gastrointestinal tract is complex due to the varying pH, the protective mucus layer and the presence of digestive enzymes (Kermanizadeh et al., 2021). This is further complicated by the fact that the different (and changing) physiochemical properties of the NMs influence their cellular interactions and uptake. It is believed that the majority of ingested NMs are rapidly passed through the gastrointestinal tract and lost via the faeces with a translocation of typically <1% of the administered dose (Kermanizadeh et al., 2021). Translocated NMs have been reported to accumulate in the liver, spleen and other organs (Kreyling et al., 2017). Nevertheless, many knowledge gaps exist including the effects of NMs on gastrointestinal tissues and microbiota (Bouwmeester et al., 2018).

Aluminum NMs are a feasible example due to their complexity. Based on an *in vitro* study of intestinal barrier crossing using Caco-2 monolayers with or without HT9-MTX mucus cells as well as a tri-culture system with M cells it was concluded that transport of Al^0 and Al_2O_3 NMs was absent. Nevertheless this conclusion should be taken with caution due to technical issues (Sieg et al., 2018). This study outlined that Al^0 and Al_2O_3 NMs were more prone to be found inside intestinal cells than the ionic form $AlCl_3$. However, *in vivo* data with the same aluminum particles showed a rapid absorption and systemic distribution after 3-day oral exposure of all Al forms tested (Krause et al., 2020). Furthermore, irrespective of the tissue, higher Al concentrations were found with Al_2O_3 NMs compared to Al^0 NMs, despite the fact that Al_2O_3 NMs contained only 50% of aluminum present in Al^0 NMs. The findings confirm challenges in the extrapolation of NMs crossing from *in vitro* models to the *in vivo* situation, as well as the difference of behavior even if the tested materials possess a similar chemical composition. The identified differences between Al^0 and Al_2O_3 NMs demonstrate that both particle agglomeration shape and surface composition could be key factors for Al biodistribution and accumulation. Recently, investigation of the genotoxicity of the three aluminum forms showed that only Al_2O_3 ENMs induced DNA damage in bone marrow while no effect was detected in duodenum, liver, kidney, spleen and blood (Jalili et al., 2020).

Currently, NMs are not added to food intentionally. However, the first Novel Food in nanoparticulate form, iron hydroxide adipate tartrate, was recently evaluated as safe by the European Food Safety Authority (Turck et al., 2021). This food supplement falls into the major area of potential nanotechnology applications in the food sector. Nanosized and nano-encapsulated food additives and supplements are described in more detail in the following chapter (Chaudhry et al., 2017). It should be mentioned that all foods containing ENMs require authorization as described in the Novel food regulation including a risk assessment by EFSA. EFSA has developed specific guidelines regarding the risk assessment of NMs and small particles to be applied in the food and feed chain (Committee et al., 2021a; Committee et al., 2021b). A lot of focus has been on food additives that can release or contain a fraction of particles in the nanosize range, such as E171 (titanium dioxide), E172 (iron oxides) or E551 (silicon dioxide) (Fig. 2, B-D).

In the ongoing re-evaluation of the safety of permitted food additives by EFSA, the presence of small particles is considered and the conventional risk assessment, if necessary, complemented with nano-specific considerations. For example, a detailed risk assessment was performed for E171. A concern for genotoxicity could not be ruled out, and given many uncertainties, the EFSA Panel concluded that E 171 can no longer be considered as safe when used as a food additive (Committee et al., 2021b).

ENMs could theoretically be released from food contact materials, especially in the case of non-intended use conditions, such as scratching. In addition, they could enter the food chain in the form of environmental contaminants as described in the following chapter. Incidental NMs could, e.g., be formed during preparation and production of food or originate from other anthropogenic activities. Examples of naturally occurring (inorganic) NMs are biogenic silica and soil silicates. These diverse sources of NMs make it difficult to analyze food for intentionally added ENMs and food additives containing small particles.

2.2. Food contact materials

ENMs have found successful application in food contact materials. A comprehensive list of materials used in the EU can be found in the Community List of the plastic FCM regulation (The European Commission, 2011). The approval of these ENMs is based on a case-by-case risk assessment. A detailed discussion is found for example in Rasmussen et al (Rasmussen et al., 2019) Among the most used materials are carbon black and silicon dioxide which have been embedded in FCMs for decades (Störmer et al., 2017). Furthermore, nano-sized titanium nitride (nano-TiN, typically 20 nm) is used. The addition of nano-TiN enables a

better heat transfer into the polymer during stretching of, e.g., polyethylene terephthalate (PET) preforms into bottles compared to the plain polymer (EFSA, 2012). As a consequence, production rates can be increased, and energy saved. The laboratory scale preparation of such a nanocomposite with polyethylene as host polymer using a master batch of nano-TiN (20 nm) in polyethylene is shown in Fig. 3B-D.

2.2.1. Release pathways and analytical challenges of ENMs in FCMs

Over the last decade, the question whether ENMs such as nano-silver or nano-TiN can be released from polymer nanocomposites for food contact use has been intensively discussed, sometimes controversially (Szakal et al., 2014; Bi et al., 2018). A main point of criticism is that in most of the published migration studies nano-silver-polymer composites were used as a model system. Silver is a known chemical redox system and is, depending on its chemical environment, able to reversibly change its oxidation state from zero (elemental silver) to +1 (dissolved ionic silver). Thereby artefacts in migration experiments may arise. Ionic silver could migrate under test conditions and later precipitate as elemental nanoparticulate silver. This may already occur during the migration test phase itself or during subsequent sample preparation steps. Precipitation of other nanoparticulate insoluble silver species (such as Ag_2S) is also possible. These are then detected either by electron microscopic imaging or spICP-MS. In addition, artefacts may occur due to the choice of non-ideal experimental migration test conditions, such as cutting the test sample into pieces followed by complete immersion in the food simulant.

In principle, three potential release mechanisms can be considered for ENMs from food contact polymers. I) The diffusion within the polymer to the food contact surface and the detachment from there into a food; II) the loss from the polymer matrix when it is degraded or stressed by intensive interactions with its environment, and III) detachment from the surface when the ENMs are not fully embedded in the polymer matrix or in the case of surficial positioning (coating).

A number of conclusive publications in the period 2017 to 2020 established that NMs do not exhibit the properties that are the determinants for migration of conventional chemicals (Störmer et al., 2017; Poças and Franz, 2018). This is due to changed physico-chemical and structural characteristics of NMS. In fact, numerous studies,

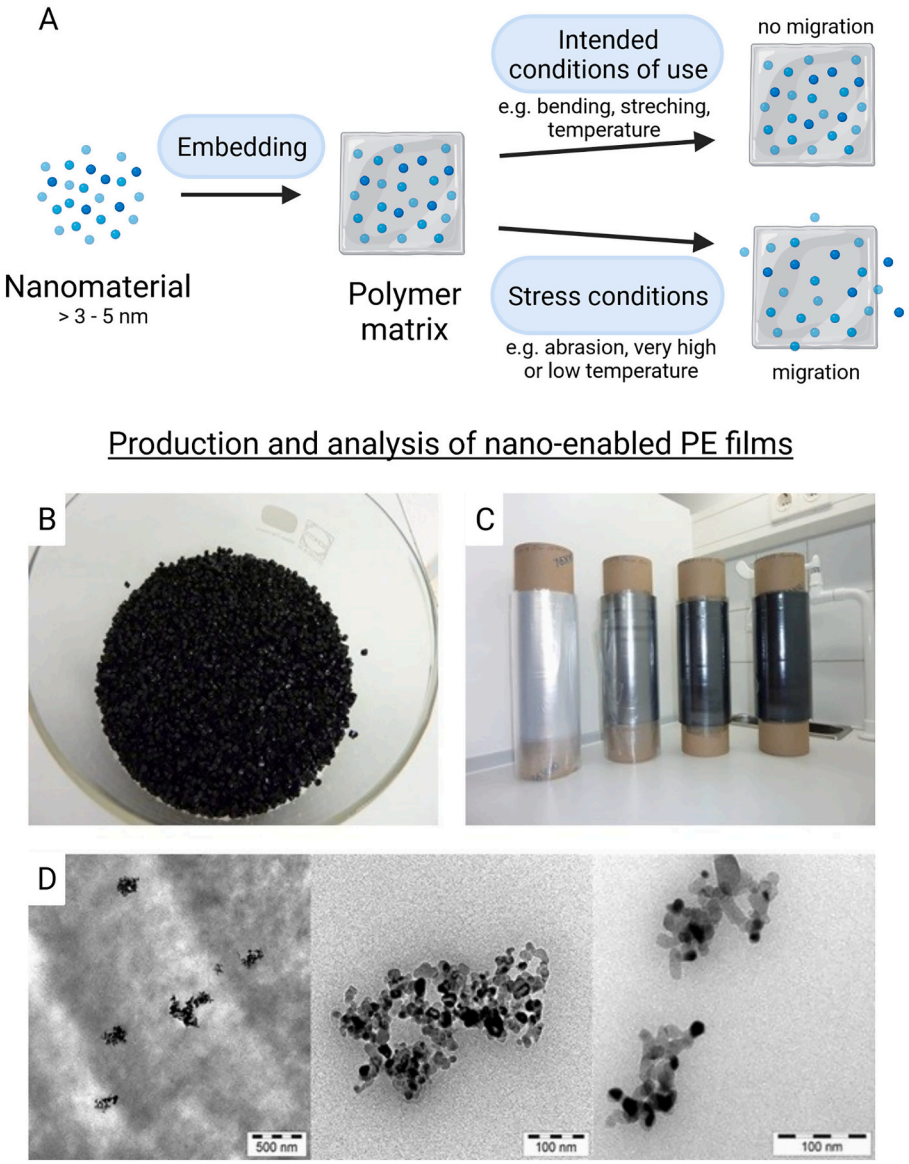

Fig. 3. (A) Schematic representation of possible migration scenarios; (C) Polyethylene films (d = 60 μm) with different nano-TiN levels (0 ppm, 100 ppm, 500 ppm, 1000 ppm) produced using a masterbatch of nano-TiN (20 nm) in polyethylene (B); (D) TEM images of a polyethylene film with nano-TiN incorporated (size of primary nanoparticles: d = 20 nm). Modified with permission from (Bott and Franz, 2014). Copyright 2022 Elsevier. *Created with BioRender.com.*

supported by theoretical considerations and modeling, have shown that NMs >3–5 nm in diameter are not able to diffuse under normal conditions of use, when fully embedded in a polymer matrix (Störmer et al., 2017). Consequently such NMs cannot migrate out from such matrices based on diffusion mechanism (Bott and Franz, 2014; Franz and Welle, 2017). It should be noted that typically NMs in the size range below 5 nm are not used in polymer nanocomposites (Störmer et al., 2017).

The question whether NMs can be released from nanocomposites if they are not completely embedded in the polymer matrix or if stress conditions can affect the polymer surface can be addressed by means of a suitable test design (Fig. 3A). Such abrasion tests can simulate the particular in-use situation of a packaged food and can address the potential influence on material ageing. Mechanical and thermal material stress factors such bending or stretching and high temperature treatments or deep freezing-storage of food contact materials and articles can also be simulated. Usually, an abrasion test in combination with an appropriate analytical method is applied. Here, either a pure chemical analysis via ICP-MS, or, when needed, techniques such as AF4 analysis combined with multi-angle laser light scattering (MALLS) detection or ICP-MS can be applied (Bott and Franz, 2019; Franz et al., 2020).

2.2.2. Safety of nano additives in food contact polymers

The European Food Safety Authority (EFSA) has published several opinions on the safety of nano additives in food contact polymers. Taking into account the abovementioned studies, EFSA concluded for all evaluated cases that no or no significant migration of NM would occur under the intended conditions of use (Poças and Franz, 2018).

2.3. Pharmaceuticals and nutraceuticals: Controlled release from nano-sized delivery systems

In addition to the prominent use of NMs in modern medicine for the controlled delivery of drugs, NMs are also used in the food sector as nano-scaled delivery systems for functional substances. For medical purposes, the delivered functional substances are active pharmaceutical ingredients (APIs) of various chemical nature. In the food sector they are proteins, enzymes, vitamins, or other bioactive compounds. They are encapsulated in a carrier material, creating particles with diameters in the nanometer to millimeter range.

The global food encapsulation market is estimated to grow annually by 6.8% from 2016 to 2024 reaching $52.4 billion by 2024 (Kumkar, 2017). Since about 2010, interest in the design of engineered nano-sized delivery systems for food application has increased (Ezhilarasi et al., 2013; McClements et al., 2009; Choi and Kwak, 2014; Oehlke et al., 2014; Assadpour and Mahdi Jafari, 2018; Nowak et al., 2019). The possibility of increasing the low absorption or bioavailability of many compounds that are claimed to benefit human health is the main reason for this trend. At the same time, the global market for biopharmaceuticals needed in future treatment prospects in the fields of oncology, immunology, diseases of the central nervous system and infections is also growing. In Germany alone, this market is estimated at 14.6 billion € (Biotechnology Report 2021, Boston Consulting Group & vfa.bio).

2.3.1. ENMs for conservation and delivery

Novel encapsulation and formulation strategies are required to enable stable and shelf-stable dosage forms for sensitive molecules such as proteins and antibodies (Bowen et al., 2013). In addition to advantages such as the ability to mask undesirable tastes and flavors, other benefits are attributed to nano-sized delivery systems. They enable the protection of the encapsulated compound from environmental factors such as pH, moisture, heat, light, chemical and biological degradation (Fang and Bhandari, 2010). Furthermore, they can assist to control interactions of the encapsulated compound with the matrix and the release of the encapsulated compound. On top, they can improve dispersion and suspension of insoluble compounds, and incorporate the delivery system into solid dosage forms without affecting their handling and the appearance of the final product (Wintermantel and Ha, 2002).

Nano-sized delivery systems can be produced by ionic or covalent crosslinking of biopolymers, emulsification, self-assembly, spray-drying and comminution of larger materials (Oehlke and Greiner, 2014). When applied to food or medicine, nano-sized delivery systems must fulfil certain criteria. All substances used must be food-grade, generally regarded as safe (GRAS) or listed by the appropriate regulatory authority such as the United States Food and Drug Administration (U.S. FDA) and the EMA or EFSA (Danhier et al., 2012). Furthermore, compatibility of the delivery system with the matrix material needs to be considered. In addition, the delivery systems must exhibit high loading capacities as well as acceptable release properties (Bourganis et al., 2018; Ahmadi et al., 2020). Moreover, it should not affect the sensory perception of the consumer, e.g., haptics of the food or chewing sensation. Finally, availability and cost efficiency are important for an economic use of nano-sized delivery systems in the food and the medical sector.

In the medical sector, the possible routes of application additionally vary, which in turn implies further specific requirements for the nano-sized medical delivery system. In addition to the oral route of administration, which is the primary route for enteral use, the requirements are similar to those for delivery systems for the food sector. In contrast, medical delivery is also administered parenterally, and parenteral application includes a variety of routes (MacGregor and Graziani, 1997; Torgerson, 2013). The most common parenteral applications are subcutaneous, intramuscular, intravenous and intrathecal. While subcutaneous injections are rather simple and also suitable for home care, intramuscular and intravenous administration are common but cannot be self-administered in most cases. In fact, intrathecal administration is the most specific administration and has low patient compliance (Hammond, 1988; Hetherington and Dooley, 2000). Therefore, it is used only in severe diseases, such as cancer or neurological disorders. A relatively new route of application, which is mainly still being addressed in research, is intranasal application. Its advantages are: (i) the ability to directly reach the brain and central nervous system (CNS) by bypassing the obstructive blood-brain barrier, (ii) consequently, to reach higher concentrations of biopharmaceuticals in the brain and CNS, (iii) to reduce severe side effects associated with biopharmaceutical applications, e.g., intravenously, and (iv) to improve patient compliance by replacing intrathecal application (Gänger and Schindowski, 2018; Keller et al., 2022). After each of these applications, the release of the encapsulated compounds can occur through diffusion or degradation of the nano-sized delivery system by for example digestive enzymes (Bruschi, 2015). Furthermore, changes in parameters such as pH, temperature, and moisture could trigger the release and result in beneficial availability at the target site, e.g., in the colon or at the olfactory cleft enabling non-invasive drug administration to the CNS efficiently via Nose-to-Brain Delivery (Bourganis et al., 2018; Ahmadi et al., 2020; Gruber-Traub and Ullrich, 2018). The release can also be dependent on variables such as shape and dimensions of the delivery system, as well as its chemical composition and chemical identity of the compound to be encapsulated. The chemical composition of the delivery system can trigger either specific binding mechanisms or even membrane opening, such as, chitosan nano- and micro-particles. The specific examples for NMs presented here (Fig. 4A) aim to increase the bioavailability of high molecular-weight antibodies in the brain and CNS following intranasal application, while stabilizing these sensitive biologics in the formulation by protecting them from light and heat. Fully encapsulated antibodies are released into the olfactory mucosa tissue either simply by increased humidity in the nose and pinocytosis (Fig. 4B) or stimuli-responsive opening of intercellular bonds (Fig. 4C) such as opening of tight junctions through chitosan (Spindler et al., 2021). In addition, NMs equipped with specific binding sites facilitate targeting of various locations in the body (Fig. 4D), e.g., the CD20-epitope on pro-inflammatory B-cells to block autoimmune reactions. As a consequence, both NMs and large

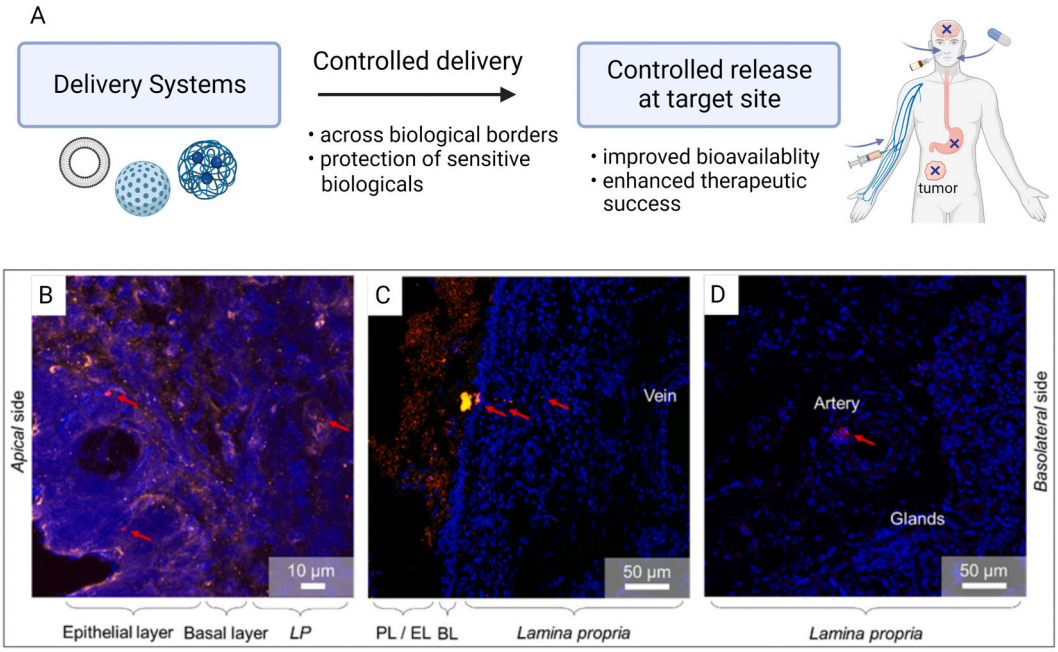

Fig. 4. (A) Benefits of nano-sized delivery systems; *Created with BioRender.com.(B-D)* Confocal Laser Scanning Microscopy images. Poly(lactic-*co*-glycolic acid) (PLGA) NMs (red) are taken up into porcine olfactory mucosa explants enabling Nose-to-Brain-Transport. (B) Pure PLGA NMs (red) are taken up intracellularly into the epithelial cell layer (EL). Nuclei (blue, 4′,6-Diamidin-2-phenylindol/DAPI), *Zonula occludens* protein 1 (amber), magnification 630×. (C) Chitosan-coated (yellow) PLGA NMs (red) are taken up and transported paracellularly into deep layers of the *lamina propria* (*LP*) enabled by tight junction opening. Nuclei (blue, DAPI), mag. 200×. (D) PLGA NMs (red) equipped with a CD20-binding monoclonal antibody enable CD20-targeting and therewith may, for example, block pro-inflammatory B-cells in the treatment of autoimmune diseases. Nuclei (blue, DAPI), mag. 200×. PL: Particle layer, BL: Basal cell layer. *BioRender.com* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules, e.g., proteins and antibodies, can be efficiently transported across biological barriers improving their bioavailability and may enhance therapeutic success. Additionally, the distribution, solubility, and diffusivity of the encapsulation material(s) and surrounding media, their localization within the delivery system as well as encapsulation load and loading efficiency can have an impact (Fathi et al., 2012). Various lipid-, protein- or polysaccharide-based nano-sized delivery systems are already described including solid lipid nanoparticles, biopolymer particles, micelles or liposomes (Ezhilarasi et al., 2013; McClements et al., 2009; Choi and Kwak, 2014; Oehlke et al., 2014; Assadpour and Mahdi Jafari, 2018; Nowak et al., 2019).

2.3.2. *Knowledge gaps and future research needs for successful application*

In general, nano-sized systems designed for the delivery of minerals, secondary plant metabolites, and other bioactive compounds have been described in the literature. Experimental evidence for an enhanced bioavailability up to a factor of 10 of these compounds when using nano-sized delivery systems was provided by *in vitro* as well as *in vivo* studies (Oehlke et al., 2014; Seju et al., 2011). Prevention of degradation, improved solubility and controlled release of the encapsulated compound in the gastrointestinal tract as well as facilitating its transfer through the intestinal wall are mechanisms responsible for an increased bioavailability (Oehlke et al., 2014; Oehlke and Greiner, 2014).

However, the majority of the nanoscale delivery systems studied were not examined in a model matrix, but in water (Oehlke and Greiner, 2014). Since release properties of the delivery systems might be significantly affected by interactions with the matrix, the findings of the above-mentioned studies might not be transferable to real food or *in vivo* systems. Delivery systems that are stable against gastric digestion and have slow release kinetics of the encapsulated compound are of particular interest with regard to improving absorption. In addition, particle size, the physical state, and surface properties of the nano-sized delivery system are key parameters to be controlled in order to obtain an improved absorption of the encapsulated compound. Before using nano-sized delivery systems for food or medicinal products, potential health risks associated with their use must be identified. Particle distribution in the target tissue, biocompatibility, biodegradation, and possible immune responses need to be taken into account (Spindler et al., 2021). However, the composition of these nano-sized delivery systems is mainly bio-based or at least completely digestible, which is generally regarded less critical than inorganic NMs lasting extended time periods in the body. An increased bioavailability of the encapsulated compound might further lead to a need to establish new accepted daily intakes for these substances. Furthermore, there is still limited knowledge of the behavior, fate, and effects of orally administered nano-sized delivery systems (Roger et al., 2010; Powell et al., 2010).

Additionally, differences in the digestion of the nano-sized material compared to its individual building blocks are an important issue. Whereas the uptake of intact delivery systems may be critical, it is difficult to predict potential adverse effects to human health due to possibly altered tissue and organ distribution of the encapsulated compound. On the one hand, those systems aim to be completely resorbable and digestible, due to their composition and design. In contrast, small changes in the formulation and interactions with the matrix might affect their resorbability in a significant manner. Studying nano-sized delivery systems in real foods and their behavior after administration *in vivo* are challenging due to the limitations in the available analytical methodologies.

2.4. *Medical devices*

Nanotechnology has become indispensable in the field of medical devices. New ceramics for dental fillings or screws for dental implants often consist of sintered nano-powders whose advantageous properties are required for their production. However, human exposure to NMs can occur by deliberated or incidental in situ release of (nano)particles from implanted medical devices. Such implants comprise, e.g., visual prosthesis, cardiovascular stents/prostheses, dental implants, osteosyntheses

materials, and orthopedic implants. The intended and controlled release of ENMs from medical implants to promote favorable local tissue response represents a significant research field in regenerative nanomedicine (Kumar et al., 2020; Nelson et al., 2020a). Prominent examples of controlled NM release from nanotechnology-enabled medical products are antibacterial ENM coatings to prevent peri-implant infection and nanoparticle-eluting cardiovascular stents to facilitate sustained release of pharmacologic agents (Chouirfa et al., 2019; Tan et al., 2012). However, also specific concern has emerged with regard to incidental release of in situ generated NMs from different implanted medical devices originating from wear processes (Olmedo and Jacobi-Gresser, 2021; Eltit et al., 2019).

2.4.1. *Release of nanoparticles from orthopedic implants*

A possible source of this type of NM release are orthopedic implants in the human body, one type being joint arthroplasty, which may pose acute and long-term risks. Major factors of material breakdown are the implant per se, the combination of implant materials and the type of implant component junction. NMs released from articulating components (two components are tribologically matched to restore motion) of arthroplasty implants are mainly associated to tribocorrosion and edge-loading. In contrast, material degradation in modular, non-articulating components result from crevice/fretting corrosion (Mischler and Muñoz, 2013; Eliaz, 2019). Especially hip implants with cobalt-chromium-molybdenum (CoCrMo) containing metal-on-metal (MoM) articulations or modular stems are associated with the local release of metal-containing NMs (Xia et al., 2017) (Fig. 5A). Due to the comparatively poor survival of MoM implants (Smith et al., 2012a; Smith et al., 2012b), the main focus of risk/benefit evaluation and regulatory challenges has been on these implants (Sedrakyan, 2012).

Ex vivo analyses of the peri-implant membrane from MoM endoprostheses indicate distinct local exposure to cobalt and chromium containing NMs (Morrell et al., 2019; Rakow et al., 2016). Xia and colleagues additionally reported exposure to titanium and vanadium containing particles in the peri-implant membrane of modular non-MoM endoprostheses (Xia et al., 2017). Current histopathological consensus on the classification of inflammatory changes in the peri-implant membrane indicates that nano- and microscale particles are also released from other than CoCrMo containing articulations or non-articulating implant components (Perino et al., 2018). The particle spectrum in terms of (multi-)metal composition, size, shape and quantity strongly depends on the implant status, possible previous ipsilateral implantations and the in situ time of the implant components (Schoon et al., 2017a; Xia et al., 2017). Cobalt, chromium, molybdenum, titanium, aluminum, vanadium, niobium, tantalum, nickel, iron and zirconium are used in orthopedic implants as pure metals, metal oxides, alloy constituents, or as contrast agents in orthopedic cement. They are therefore most relevant to multi-element analyses and particle constitution. Local metal level quantification and particle characterization have mainly been performed in ex vivo synovial fluid and the peri-implant tissue.

The physicochemical characterization of incidentally released NMs from medical devices including the quantification of elemental particle composition within human tissue samples is inevitable for the assessment of particle reactivity. Particle characterization within the tissue of interest, i.e., without prior particle isolation, has considerable advantages due to the unaltered matrix. This is especially true when investigating oxidatively less stable NMs, such as those released from CoCrMo alloys. Available methods are, e.g., electron microscopy-based techniques (SEM, TEM, STEM), which have the limitation of prior sample processing including embedding and sectioning. X-ray fluorescence (XRF) spectroscopy represents a viable alternative allowing for multi-element tissue mapping. Based on the enormous x-ray intensities provided by synchrotron sources, synchrotron-XRF spectroscopy has become a powerful tool for assessing elemental distribution and speciation with sensitivities down to the ppm range at the nanoscale. This is

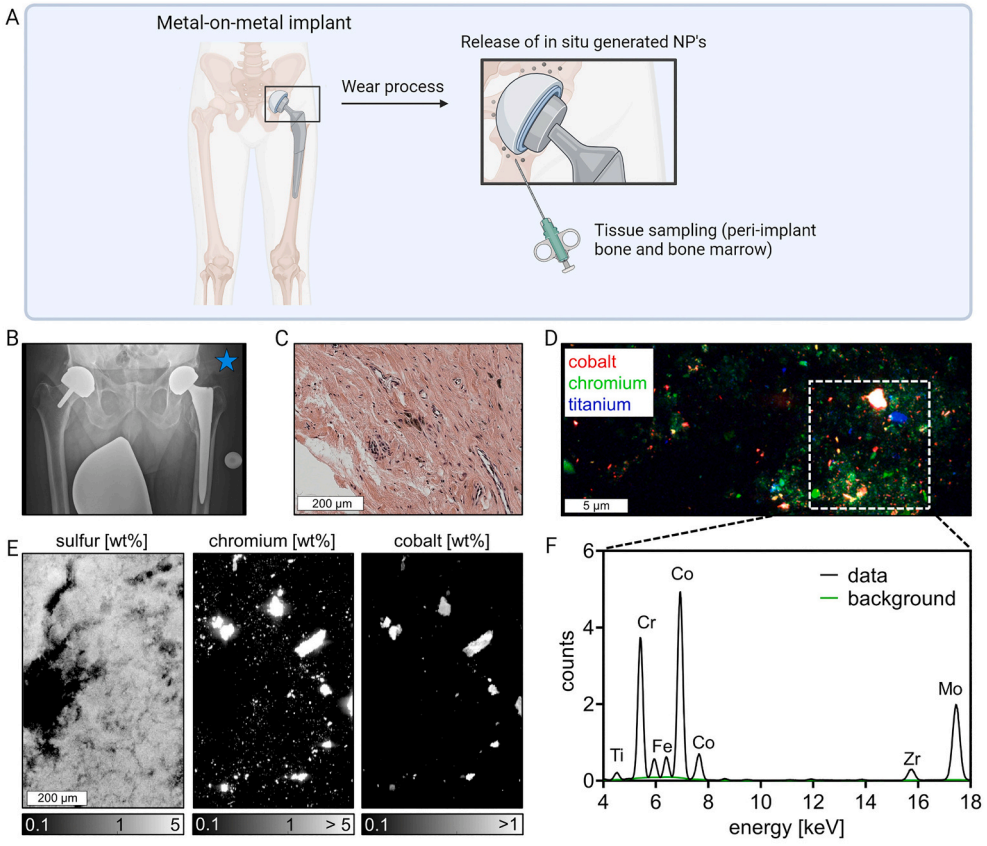

Fig. 5. (A) Local release of metal-containing NMs into implant site; (B) Spatially resolved metal detection in periprosthetic soft tissue from a patient undergoing revision surgery of a large head metal-on-metal hip endoprosthesis eight years after primary implantation due to osteolysis, pseudotumor formation and elevated systemic cobalt and chromium levels showing exposure to metal-containing particles; (B) Pre-revisional radiograph. The blue asterisk indicates the side of intraoperative sample extraction; (C) Hematoxylin and eosin staining of the sampled periprosthetic tissue indicates particle-laden macrophages; (D) Quantitative elemental concentration maps of the adjacent unstained section obtained through XRF at a spatial resolution of 3 µm indicates exposure to chromium and cobalt containing particles within periprosthetic tissue (sulfur); (E) Qualitative RGB (red: cobalt, green: chromium, blue: titanium) nano-XRF map of the same tissue section with a spatial resolution of 60 nm indicates exposure to nano-, submicron-, and micron-sized particles; (F) Nano-XRF-spectrum of micron- and nano-sized particles (dashed line, d) clearly indicates multi-element particle exposure. *Created with BioRender.com.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of special interest for life science applications, e.g., related to implant-related NMs (Morrell et al., 2019; Nelson et al., 2020b). Spatially micron- and nano-resolved metal quantification by synchrotron-based XRF of inflamed periprosthetic soft tissue allows to determine physico-chemical characteristics of released metal containing particles (Fig. 5B-F).

2.4.2. Health effects by nanoparticles from orthopedic implants

Particle-induced inflammatory pathologies of peri-implant tissue are histo-pathologically characterized by chronic innate immune responses, or less prevalently, by acute adaptive toxic or allergic hypersensitivity (Krenn and Perino, 2017; Perino et al., 2021). The patho-mechanism of the clinically most dominant particle-related peri-implant pathology, aseptic osteolysis, is linked to this inflammatory peri-implant environment (Gallo et al., 2013). *In vitro* studies suggest direct exposure-related diminished bone metabolism due to alterations of osteoblastic cells towards diminished bone matrix formation and enhanced bone resorption (Rakow et al., 2016; Andrews et al., 2011; Goodman et al., 2006). A recent study supports these *in vitro* results by indicating that a peri-implant membrane does not isolate the implant components from bone and bone marrow. This study shows that human peri-implant bone and bone marrow are significantly exposed to metallic micro- and nanoparticles (Schoon et al., 2020). Notably, *in vivo* generated metallic NMs can dissolve locally to a certain extent (Morrell et al., 2019; Schoon et al., 2017a). The particles' solubility might depend on the local cellular and non-cellular environment, elemental composition, oxidative state, and the capability to bind to local tissue matrix. The relation between solubility and the capacity to systemically distribute is shown by the elevation of particularly oxidative less stable metals such as cobalt, chromium, vanadium and nickel in the circulation of patients (Newton et al., 2012; Catalani et al., 2013). Neurological, cardiovascular, and endocrine systemic effects have been reported for patients with MoM hip implants and comparatively high systemic cobalt levels (Bradberry et al., 2014; Cheung et al., 2016; Catalani et al., 2012).

2.4.3. Knowledge gaps and future needs for medical devices

It should be noted that an in situ MoM hip implant was the only clinical indication for systemic cobalt and chromium level quantification. To assess exposure to other relevant metals, multi-metal quantification in whole blood of a broader arthroplasty clientele is needed. Recently, systemic multi-metal quantification has gained increasing acceptance in clinical routine diagnostics (Rakow and Schoon, 2020). Moreover, multi-metal quantification should be performed in other systemic compartments to assess potential compartment specific accumulation of metals. Case reports revealing elevated cobalt levels in cerebrospinal fluid suggest cobalt transfer to the central nervous system (Tower, 2010; Rizzetti et al., 2009; Steens et al., 2006). The advanced age of patients and age-associated increased prevalence of neurological deficits (e.g., polyneuropathy in type 2 diabetes mellitus) may potentially mask adverse effects of metal exposure and may lead to underestimation of neurotoxic risks.

Multi-metal quantification and particle characterization in various human ex vivo tissues and fluids of an adequate number of patients will help to gain new insights regarding local and systemic toxicokinetics of in situ generated metallic NMs. *In vivo* NM release from orthopedic implants is a real and unabated topical exposure scenario in humans (Schoon et al., 2017b). Although such particles do not originate from nanotechnology, risk assessment of NMs may benefit profoundly from the increasingly detailed and standardized methods for risk assessment of incidentally formed debris.

2.5. Spray products and aerosolized ENMs

In addition to potential oral exposure through products such as food or direct systemic availability through *in vivo* release from orthopedic implants, products such as nano-enabled sprays generate aerosolized ENMs that can lead to consumer exposure via inhalation. There are recent studies linking ENM aerosols released from toner-based devices such as printers and photocopiers to potentially high exposures and to toxicological and potentially serious health consequences (Setyawati et al., 2020; Jia et al., 2022). Cohort studies in humans show an induction of chronic systemic and upper airway inflammation as well as exposure associated effects on the serum metabolome (Jia et al., 2022; Bello et al., 2021).

To link exposure to particles released from nano-enabled products to toxicology, valuable integrated methods are described in the literature. These include standardized protocols for evaluating the release and toxicological effects of ENM release throughout the life cycle (Pal et al., 2015). In addition to the release of intentionally added ENMs, depending on the spray parameters and the formulation being sprayed, NMs may also be generated during spraying and subsequent drying of the aerosol, even if none were present in the formulation (Nørgaard et al., 2009). These sprays are often of high complexity and adverse effects on the lung have been described in the literature (Nørgaard et al., 2010).

Besides inhalation exposure, incidental dermal contact with ENM-enabled spraying products may occur. However, possible adverse effects are likely reduced due to low dermal absorption hence lower systemic availability (Kim et al., 2015). Therefore, although spraying products, such as biocides or coatings, may lead to dermal exposure the inhalative route is generally considered to pose a higher risk for these release scenarios. In the case of, e.g., sunscreens containing NMs such as TiO_2, regulatory committees such as the EU's Scientific Committee on Consumer Safety recommend against using applications with unnecessary spray methods (Pastrana et al., 2018). Here, the dermal absorption is negligible whereas the inhalation is bearing hazard.

Further scenarios for potential exposure to airborne ENM, which will not be discussed here, include processes where handling and especially manipulative or destructive treatment (e.g., drilling, sanding, sawing, or incineration) of ENM-enabled products or composites is involved (Gomez et al., 2014; Lee et al., 2020). In general, the release of ENM-containing aerosols can be observed in these scenarios and although they are often still bound to matrix material or found as agglomerates, details depend on material and process in each individual case. Therefore, inhalation exposure and possible adverse effects also need to be assessed case by case. This chapter is focusing on specific analytical issues of spray products and certain perspectives of inhalation toxicity of aerosolized ENMs regarding their dose metrics for risk assessment.

2.5.1. Analysis of nanomaterial-enabled spray products and identifying knowledge gaps

The growing use of ENMs as constituents of spray products such as cosmetics, coating or cleaning products increases the likelihood of human exposure via inhalation (Bierkandt et al., 2018). Only insufficient information is available on release and deposition behavior of such formulations and about their toxic potential. Adverse effects associated with aerosol inhalation have been described to be influenced by the size as well as the shape of ENMs (Sukhanova et al., 2018). Among other parameters, such as breathing pattern, these factors influence particle deposition in the respiratory system. Moreover, the aerosol toxicity of sprayed ENM-enabled formulations is affected by their constituents and the quantity. Besides being potentially toxic, these excipients may alter, e.g., the ENM's surface or agglomeration state. In consequence, particle reactivity or cellular uptake may be altered (Bian et al., 2011; Mehta et al., 2009). Thus, characterization of spray formulations and the state of ENMs in the formulation, e.g., agglomeration or surface coating in solution as well as after aerosolization, is an essential first step (Losert et al., 2014; Jiang et al., 2009). Additionally, constituents of spray formulations can greatly affect the spraying behavior. The viscosity or surface tension can be altered by solvents, additives, and excipients, thus affecting the droplet size of the generated aerosol which in turn impacts the distance the aerosol travels before settling (Sirignano, 2010). To achieve realistic scenarios under experimental conditions, the

aerosolization process via spraying should mimic real life products as far as possible. This can be achieved, for example, by using commercially available spray cans and nozzles and taking into account the respective spraying methods (e.g. pump sprays vs. pressure gas sprays). Furthermore, the characterization of the whole emission chamber including reliability and reproducibility is necessary. For spray applications the toxicity of all constituents should be evaluated (Pearce et al., 2019). Different spray scenarios, varying in spray duration, pulse frequency and number, can be tested during exposure studies, e.g., on lung cells, to mimic realistic conditions. The applied formulation and experimental setup should be carefully considered and chosen to be as realistic as possible in order to enable a reliable assessment of risks based on the inhalation of ENM-enabled products (Osmond-McLeod et al., 2015).

In order to understand the complex interactions between ENMs, spray formulation, spraying device and spraying regime, as well as their effects on generated aerosol and its toxicity these parameters have to be studied simultaneously. The international NANOaers project investigated ENM-enabled (CeO_2- and Ag-ENM, each in different particle sizes) coating sprays. Part of the project was the investigation of the toxicity of the components by *in vivo* and *in vitro* tests and the physicochemical characterization of the formulations. In addition, aerosol formation and behavior during spraying as well as toxicity of the generated aerosol using *in vitro* air-liquid interface cell exposure were investigated. To enable the investigation of the individual parameters and still remain close to the conditions of consumer sprays, the complexity of the spray formulation was gradually increased. This also applies to the respective characterization. A special spraying device and spray chamber were employed to enable controlled aerosol generation and characterization. The results showed that slight changes at different levels, e.g., variations of the formulation or the spraying regime, could lead to increasing differences in the chain of events: 1) the formulation altered particle agglomeration (or in the case of Ag-ENM dissolution) and spraying; 2) which affected aerosol droplet size and ENM release; 3) similarly, the spraying device and parameters affected aerosol droplet size and released quantity; 4) the ENM size affected the aerosolization efficiency; 5) the aerosol characteristics, again, influenced the aerosol transport range and thus the cell exposure; and 6) the different constituents for formulation and the ENMs have different intrinsic toxicity. All in all, no trivial answer could be given to the toxicity of the spray agents studied. However, it was possible to identify factors that contribute to the toxicity. These include possible interaction between ENMs and surface-active additives and high toxicity of these surface-active substances. Although this project looked only at certain points during the use of only one spraying product it is clear that these complex interactions have to be taken into account in future studies.

2.5.2. Inhalation toxicity and dose metric for risk assessment

Risk assessment of NMs relies on dose-response relationships obtained for different organisms under relevant exposure scenarios. Typically, this involves analysis of toxicological, epidemiologic and, for medicinal products, clinical studies utilizing data from *in vitro* cell and *in vivo* animal models as well as human health records. Historically, inhalation has been considered the most relevant portal of NM entry for human health (Greim and Snyder, 2018; Oberdörster and Kuhlbusch, 2018). For chemicals in general, mass has been used as the preferred dose metric. However, for NMs, to our knowledge, there is no toxicological pathway that is mechanistically driven by mass. This has led to the misconception that nanoparticles are generally more toxic than microparticles. While there are nano-specific aspects related to increased hazard potential (e.g., a higher propensity for cellular uptake and migration across biological barriers), the size-induced toxicity is often overestimated because a toxicologically inappropriate dose metric such as mass is used. Since smaller particles of a given type of material have a larger mass-specific surface area than larger ones, NMs typically have a higher mass-specific toxicity than microparticles. Nevertheless, if another toxicologically unrelated dose metric such as number of (primary) nanoparticles would have been chosen as dose metric, nanoparticles would appear less toxic than microparticles, since smaller particles have less surface area per particle than larger ones (Schmid and Stoeger, 2016). This highlights the need for identification and adoption of the toxicologically most relevant dose metric.

Moreover, many dose-response relationships are presented in terms of exposure concentration such as NM mass (number or surface area) per volume of carrier medium ($\mu g/m^3$). As induction of toxicity requires direct contact between toxin and (lung) tissue, the toxicologically more relevant dose measure is the cell-delivered dose. For chemicals, the exposure concentration is generally a good surrogate for the cell delivered dose. Molecules are highly diffusive and can therefore reach the lung tissue rapidly not only during inhalation in the lung but also in *in vitro* cell culture models. However, migration of nano−/micron-sized particles in media such as air or cell culture medium is much slower and – most importantly – highly dependent on particle size and effective density. Hence, only a fraction of the available particles reaches the lung tissue or cell culture model rendering exposure concentration a poor predictor of tissue−/cell-delivered NM dose (Hinderliter et al., 2010). As an alternative to exposure, tissue-delivered dose normalized to some tissue-scaling parameter (e.g., $\mu g/cm^2$-tissue or μg/g-lung) has been proposed, since this normalization allows for allometric scaling of dose-response curves from *in vitro* to *in vivo* models and even to humans as required for risk assessment (Schmid and Cassee, 2017).

In a previous report, particle agglomerate volume has been suggested as the adequate dose metric to derive reference values, while surface area was found inadequate (Laux et al., 2017). However, as shown by Tentschert et al. for nano-CeO_2, conversion of the typically measured mass into volume requires knowledge of the densities of NM agglomerates *in vivo*. It should be noted, that the agglomerate density can vary considerably depending on primary particle diameter, material bulk density, and morphology. This prevents an exact prediction of lung clearance, e.g., under overload conditions and demonstrates the need for techniques that allow a reliable detection of agglomerate density *in vivo* (Tentschert et al., 2020). DeLoid et al. determined effective agglomerate densities of various NMs and their 90% sedimentation periods in *in vitro* assays. For nano-CeO_2, the agglomerate density is approx. 2.6 vs. a material density of approx. 7 g/cm^3 (DeLoid et al., 2014). These data can be helpful to get a realistic idea of the volumetric lung load.

Several additional approaches for conversion of exposure concentration into tissue-delivered dose for animal inhalation experiments and *in vitro* cell experiments under submerged and air-liquid interface culture conditions have been reported (Schmid and Cassee, 2017). It has been shown that small particles such as NMs have higher mass-specific toxicity than larger or micron-sized particles of the same material (Oberdörster et al., 2005; Waters et al., 2008). This can be attributed to the geometric increase of mass-specific surface area with decreasing particle size (mass-specific toxicity ~1/d for spherical particles). If surface area is used instead of mass as dose metric (Fig. 6B,C), this apparent nano-specific toxicity disappears for many *in vitro* and (acute and chronic) *in vivo* studies with biopersistent particles (Schmid and Stoeger, 2016; Oberdörster et al., 2005; Waters et al., 2008; Stoeger et al., 2007; Stoeger et al., 2008; Maynard and Kuempel, 2005; Cosnier et al., 2021). This suggests that surface area is the biologically most relevant dose metric for the inhalation toxicity of particles. However, there are also exceptions to this, for example when strong shape-induced effects occur for long, rigid fiber-like particles.

The metric of surface area can be rationalized by understanding that the biological matrix is only in contact with the surface of the biopersistent NMs, but not with the inner core (Borm et al., 2015). Molarity is the most relevant dose metric for soluble materials (molecular substances). Analogous to molarity, surface area is often considered, instead of mass, as the metric of choice for non-soluble, biopersistent particulate NMs. These so-called particles of *poor solubility and low toxicity (PSLT)* form a distinct class of particles. It includes a diverse set of materials such as metal oxides (e.g. TiO_2, amorphous SiO_2, Fe-oxides),

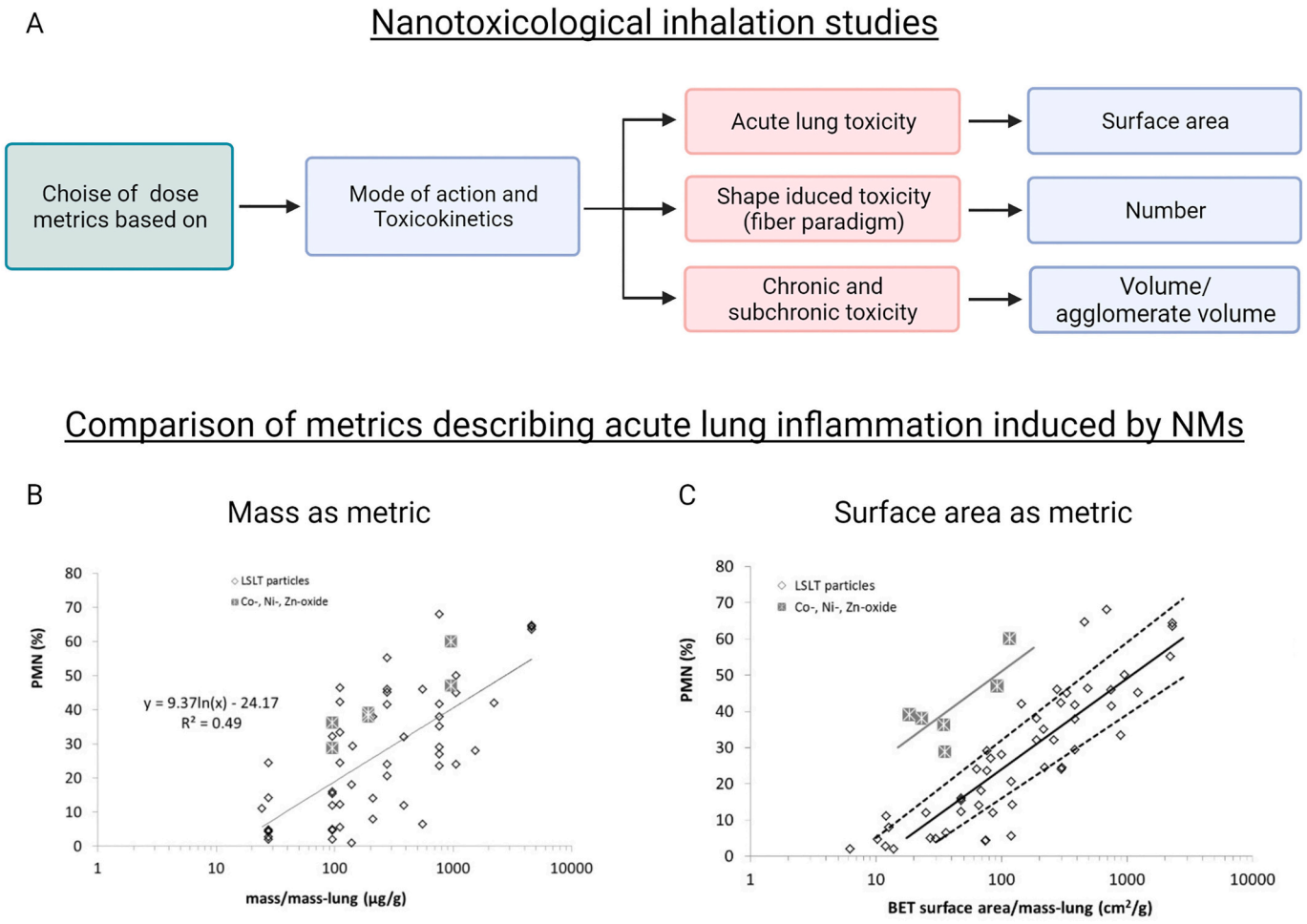

Fig. 6. (A) Overview for choice of metric in nanotoxicological inhalation studies; (B) Surface area (left panel), but not mass (right), allows for grouping of different NMs into hazard classes, namely of PSLTs and transition metal oxides (cobalt, nickel, zinc). Adapted from (Schmid and Stoeger, 2016). Copyright 2022 Elsevier. *Created with BioRender.com.*

combustion-related carbon (e.g., industrial carbon black, high- or low organic soot, spark-discharge elemental carbon) and polymeric materials (e.g., polystyrene, polyethylene).This classification is independent of the size of primary particles or agglomerates (Schmid and Stoeger, 2016; Hadrup et al., 2020).

On the other hand, the metric of volume or agglomerate volume can be rationalized by the overload concept based on the hypothesis of the mode of action of chronic lung toxicity of dust (Morrow, 1988). Here, a loss of the migratory capabilities of particle-laden, enlarged alveolar macrophages (AM) was suggested to be the cause of any increased elimination half-time of PSLT. NMs that have a higher portion of void spaces in their agglomerates, and thus a higher displacement volume at identical mass concentrations, lead to higher potency. However, lately this could not be confirmed but the overload-related increased pool-size of BAL-cells was shown to be the cause for increased elimination half-time of PSLTs. Mechanistic and kinetic-modeling has shown that the displacement volume of complex aggregated structures relative to the total volume – the pool size - of pulmonary phagocytes appears to be the most suited metric. This is independent of the corpuscular volume of the cells (Li and Pauluhn, 2018). Hence, this could have the advantage of reliably estimating the cumulative volume of phagocytosed PSLTs. Even when the effective surface area that is biologically active may change from one sub-compartmental microenvironment to another due to, e.g., protein corona formation. Furthermore, there is also evidence that the number of particles may be the most relevant dose metric for long (5 µm) and rigid high-aspect ratio (fiber-like) NMs such as carbon nanotubes (Oberdörster, 2000). On the other hand, surface area as dose metric can be used for stratification of various types of NMs into a few "hazard" classes. Each of these classes comprising numerous materials almost independent of sizes and shape (except for, e.g., long, rigid fiber-like particles). Enhanced surface specific toxicity or hazard factors related to acute pulmonary inflammation were reported for various transition metal oxides, e.g., zinc, nickel, and cobalt, yielding a 12-fold higher surface toxicity as compared to PSLT materials (Fig. 6C (Schmid and Stoeger, 2016);). It is important to note that within these hazard classes sub-classes of toxicity can be identified due to, e.g., difference in crystallinity (such as TiO_2) and/or heavy metal impurities (such as soot) (Rubio et al., 2019; Danielsen et al., 2020).

Taking these aspects into account, nanotoxicological studies should carefully choose the dose metric describing the collected data best. A decision on the most appropriate dose metrics should be based on knowledge about the mechanisms underlying the observed effect as well as toxicokinetic data (Fig. 6A). This may only be possible on a case-by-case basis or for specific groups of NMs. For surface-related modes of action which are driving acute NM lung toxicity the surface area might be the most relevant dose metric. The relevance of other dose metrics such as number is acknowledged in the context of different modes of action, namely shape-induced toxicity (fiber paradigm). The volume or particle agglomerate volume might be the most relevant dose metric for chronic and sub-chronic studies such as repeated dosing leading to inflammation and high particle lung burden (Li and Pauluhn, 2018; The MAK-Collection for Occupational Health and Safety, 2014). In these

study types the volumetric lung loads, calculated with experimentally determined agglomerate densities, allow a realistic prediction of the toxicokinetic outcome (non-overload or overload status) (DeLoid et al., 2014). However, one limitation is the lack of available validated methods for quantifying the exposure such as counting of nanofibers. Furthermore, the transfer of laboratory measurements and their conclusions to exposure measurements, where the nature of the exposure may be very different from the exposure generated in the laboratory, is a major challenge.

In summary, dose-response relationships should be reported in terms of normalized tissue-delivered NM dose such as dose per surface (or mass) of exposed tissue. Allometric scaling can then be performed in terms of tissue-delivered and tissue-normalized dose. For regulatory purposes "safe" levels of tissue-delivered dose can subsequently be converted into equivalent exposure concentrations. Surface area, number or displacement volume per volume of phagocytes are highly predictive of hazard for specific modes of action. The conversion to equivalent mass dose and mass exposure concentration will remain important for regulatory purposes as long as mass-based exposure levels are the only regulatory accepted measure for exposure control.

3. Conclusions

This review demonstrates the challenges in the fields of NM release, analysis and toxicological consideration with a focus on several NM containing product groups. These insights into the various aspects clearly show the diversity and complexity in the field of nanotechnology and nanosafety. Remarkable progress has been made in both areas, but on the other hand, future goals are being formulated to fill knowledge gaps and meet the needs for safe use. The development of more advanced detection methods and strategies provides a better basis for work that contributes to a more consistent understanding of the release potential of NMs. The latter can be converted into potential exposure data that can then contribute to risk assessment. For example analysis of human ex vivo samples of patients with orthopedic implants will help to gain new insights regarding toxicokinetics of in situ generated NMs. However, it has already been shown that *in vivo* NM release from orthopedic implants is a real and unabated topical exposure scenario in humans.

On the other hand, future developments and complexity of derived new products and new materials including advanced materials will be dealt with as challenges arise. Some applications have already found broad acceptance, for example within plastic food contact materials where EFSA has published several favorable opinions on the safe use of applications of additives in nanoform, concluding that no significant migration of NMs can be expected under the intended use conditions.

Nevertheless, several knowledge gaps have been identified and future demands can be defined dealing with NMs in food, in the form of nutritional ingredients, from natural sources, or as (part of) food additives. The knowledge gaps include the need for validated methods for the detection of NM in products such as food and emphasize a growing need for validation, standardization, and implementation of recognized testing methods and tools. This is especially important for biopersistent NM, but also biodegradable materials, about which even less is known. These would also be important for studying scenarios for novel nano-sized delivery systems in, e.g., food and their behavior after oral administration, which pose a challenge. Especially, bioresorbable and digestible NM as nano-sized delivery systems offer unique opportunities. These include delivery and release of cargo molecules to target sites of interest where conventional supplements and medicines fail. This creates a solution approach for more specific applications such as nasal delivery to the brain.

Furthermore, problems such as discrepancies between *in vitro* and *in vivo* nanotoxicological studies as well as mechanistic studies highlight that complementary approaches are required for assessing possible hazards of NMs. Comprehensive quantification and particle characterization in various biological media and relevant local microenvironments or artificial fluids such as lung lining fluid or intestinal juice are needed for the generation of reliable data on uptake and the resulting organ burden. For nanotoxicological studies several key considerations were discussed, for example the dose metric. A universal metric which is describing dose-response relationships best is still being debated. The decision on the most appropriate dose metrics should be based on knowledge of the mechanisms underlying the observed effect (s) and on toxicokinetic data. Reporting of dose-response relationships should ideally be done in terms of normalized tissue-delivered NM dose such as dose per surface of exposed tissue.

Disclaimer

The content expressed in this paper is solely the opinion of the authors and does not necessarily reflect the opinion of their institutions.

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Declaration of Competing Interest

The authors declare the following financial interests which may be considered as potential competing interests. Wendel Wohlleben is an employee of a company producing and marketing Nanomaterials.

Data availability

Data will be made available on request.

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