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REVIEW ARTICLE



# Biomedical nanomaterials: applications, toxicological concerns, and regulatory needs

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**ABSTRACT**

Advances in cutting-edge technologies such as nano- and biotechnology have created an opportunity for re-engineering existing materials and generating new nano-scale products that can function beyond the limits of conventional ones. While the step change in the properties and functionalities of these new materials opens up new possibilities for a broad range of applications, it also calls for structural modifications to existing safety assessment processes that are primarily focused on bulk material properties. Decades after the need to modify existing risk management practices to include nano-specific behaviors and exposure pathways was recognized, relevant policies for evaluating, and controlling health risks of nano-enabled materials is still lacking. This review provides an overview of current progress in the field of nanobiotechnology rather than intentions and aspirations, summarizes long-recognized but still unresolved issues surrounding materials safety at the nanoscale, and discusses key barriers preventing generation and integration of reliable data in bio/nano-safety domain. Particular attention is given to nanostructured materials that are commonly used in biomedical applications.

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## 1. Introduction

Advanced materials that operate at the nano-bio interface exhibit novel or enhanced characteristics not observed in the bulk. While these unprecedented properties of nanostructured materials make them promising candidates for diverse applications, these scale-specific properties may also trigger undesirable health or environmental consequences (Oberdörster, Oberdörster, and Oberdörster 2005). Although the health and safety risks of nanomaterials (NMs) and their effective regulation has been given a great deal of attention over the past decades (Linkov and Satterstrom 2008; Oksel et al. 2016; Savolainen et al. 2010; Yokel and Macphail 2011), science has not yet provided clear answers to the questions surrounding the safety of NMs. The main problem hampering straightforward application of standard practices to NMs is their ability to transform from one nanoform to another in different biological environments. Considering the tremendously large variety of NMs, testing and controlling the hazardous effects of each NM across a wide range of relevant physiological conditions presents a serious challenge to existing regulations.

The risk assessment process involves identification of potential hazards and evaluation of occupational, consumer, and environmental exposure to hazardous substances, while risk management primarily focuses on the selection and implementation of effective measures to control foreseeable risks. The immediate aim of regulatory risk assessment and management is to ensure the safety of industrial chemicals in their intended applications. Traditional risk management measures follow the standard hierarchy of control strategies (elimination or reduction of the hazard by design, application of engineering controls at the source, and implementation of administrative controls and other protective measures) in order to eliminate hazard or reduce exposure to acceptable limits (Manuele 2005). However, these measures rely on the bulk characteristics of chemicals and are not fully

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responsive to nano-specific issues. Moreover, the efficiency of existing risk control measures in controlling unique hazardous behavior and exposure pathways associated with NMs is yet to be proven.

Increasing evidence (Donaldson and Poland 2013) confirms that the main principles of traditional risks assessment also apply to NMs, so scientists and regulators have shifted their focus from developing a *completely new* risk assessment methodology to *modifying* existing practices to encompass the unique features of nanoscale materials. However, as NMs are very complex systems and are still a relatively new technology, tailoring existing regulations to properly address nano-scale risks is yet to be completed.

While many questions still need to be answered in nano-safety research, a growing number of NMs continue to attract attention because of the potential benefits they provide to a wide range of industries and markets. In particular, nanostructured biomaterials in medicine promise to improve many key aspects of disease prevention, diagnosis, and treatment (Kim, Rutka, and Chan 2010; Riehemann et al. 2009; Wagner et al. 2006). The nanomedicine industry is on the cusp of a major revolution and is expected to grow to $350 billion by 2025 (Grand View Research I 2017). While the growth potential of nano-enabled products in nanomedical industries is undeniably high, exaggerating potential benefits, the well-known Gartner technology hype cycle (O'Leary 2008), is as problematic as overstating potential health risks as it contributes to public distrust of nanotechnologies (Resnik 2019).

Here, we discuss potential factors hampering effective assessment of health and safety risks of NMs within the regulatory context. We start by highlighting the long-standing but still unresolved problems in nano-safety research, and the key issues leading to data artifacts and controversies in nanotoxicology. We then discuss the latest medical applications of NMs and how to assess the associated health risks given the lack of technical standards, consensus, and legal frameworks.

## 2. Ambiguities around nano

NMs are structures having one or more dimensions smaller than 100 nm, with surface to volume ratios orders of magnitude larger than bulk materials that

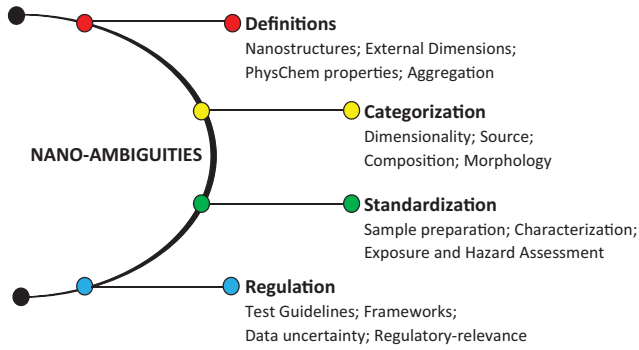


**Figure 1.** Nano-ambiguities.

may trigger specific hazardous properties. While the link between hazard and particle size alone is still unclear, the scientific evidence to date (Beaudrie et al. 2015; Falkner and Jaspers 2012; Heidmann and Milde 2013; Maynard 2014) suggests a growing controversy about the effects of long-term exposure to NMs, intensified by lack of standardized terminologies and methodologies (Gao and Lowry 2018; Kuempel, Geraci, and Schulte 2012). In particular, precise definitions and class labels are needed to avoid regional or sectoral differences in how NMs are defined, and to define sub-classes to which specific attention and regulatory assessment are more urgent. Despite many committees, reports and recommendations (Duvall and Wyatt 2011; Park and Yeo 2016; Rasmussen et al. 2016; Rauscher, Rasmussen, and Sokull 2017), the question of how to define, categorize and regulate NMs remains mostly controversial. Ambiguities surrounding nanotechnology are summarized in Figure 1.

### 2.1. Ambiguity 1: defining NMs

The problem with nano starts with definition, which have been a roadblock in deciding whether a material is a *NM* for which special legal requirements may apply. The European Commission's (EC) definition of NM, *a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm,* is based on particles' external dimensions and does not cover nanoscale internal/surface structures. The International Organization for Standardization (ISO) includes nanostructures in its definition of NMs, *material*

*with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale*, where nanoscale is defined as the size range from approximately 1–100 nm. The European Cosmetics Regulation provides an independent definition that incorporates insolubility and/or (bio)persistence, *insoluble, or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm*.

Definition problems are exacerbated by the fact that NMs cannot simply be defined by their formula and their characteristics cannot be represented by a single value. Moreover, size-dependent changes also occur in bulk properties of different materials at sizes above 100 nm. However, we stress that while conceptual ambiguity in the definition of NMs still persists (and likely always will), the definition itself serves as a guide for differentiating NMs from their bulk equivalents, not for separating hazardous materials from non-hazardous ones (Rauscher et al. 2019). Clearly, as materials properties do not undergo a sudden, dramatic change once one dimension falls below 100 nm, definitions of what constitute NMs will never be completely precise. The current definitions of different types of NMs are likely to be useful and workable in the future.

### 2.2. Ambiguity 2: categorizing NMs

Categorization of NMs is another area of research that has received considerable attention, but more work is needed (Hansen et al. 2008; Gebel et al. 2014; Godwin et al. 2015). A chemical category represents a group of chemicals sharing at least one similar physical, chemical, and/or biological feature relevant to risk assessment. Category formation through grouping chemicals with common behavior or consistent trends into distinct classes is usually intended to streamline the risk assessment and decision-making process. To date, several distinct categories of NMs have been defined according to their source (natural or synthetic), dimensionality (1D, 2D, and 3D), composition (carbon-based, inorganic, organic, and composite/hybrid), and morphology (high aspect ratio and low aspect ratio) (Dolez 2015). Despite the concerted efforts to establish science-based grouping approaches for NMs, there is still no consensus on how to apply, validate, and report nano-specific grouping concepts in a regulatory context (Mech et al. 2019). In particular, in order to group NMs according to their risk potential to eliminate the need to test every NM for every endpoint, we need improved understanding of the factors that control biological effects at the nanoscale.

When considering similar NMs as a group and applying grouping concepts for regulatory risk assessment purposes, special attention must be given to (1) justifying grouping criteria on multiple bases to validate initial category hypothesis, (2) forming information-rich categories with the highest possible number of potential members, (3) describing the logic of and data defining category formation, and (4) reporting the posterior probability that each group member follows the biological profile of reference substances.

The similarity principle has been used by chemical regulatory bodies, allowing simplified labeling of chemicals likely to have similar risk and hazard profiles. For NMs, recent developments in experimental and computational characterization of NM structures and other physicochemical properties and the relative success of read-across methods have opened the door to similar categorization (labeling) of NM with similar risk and hazard profile in the future.

### 2.3. Ambiguity 3: nanometrology and standardization

NMs can generate new toxicological risks that are poorly understood or are contradictory, leading to greater uncertainty than the well-known risks of bulk materials or industrial chemicals. Lack of standardization of experimental procedures and methods involved in the preparation, characterization, and toxicological evaluation of NMs (Roubert et al. 2016) is a major contributor to inconsistency and uncertainty in the field. This is particularly relevant for complex NMs whose physicochemical and toxicological properties are highly variable, environment-specific, and difficult to test.

Regulatory and standardization communities (e.g. Food and Drug Administration [FDA], EPA, European Chemicals Agency [ECHA 2020], ISO, and OECD) are strongly committed to development of validated methods for characterizing as-received

intrinsic properties and medium-dependent extrinsic properties of NMs, and to identify the exposure/hazard posed by NMs to humans and the environment. Compared with the measurement of pristine properties free of the influence of biological environments, assessing properties of NMs that change over time or in different biological fluids is less standardized and more technically challenging.

This ambiguity is more difficult to address as it bears on how NMs are recognized by cells and other biological systems. The 'sizes' of NMs clearly depend on the environments in which they impact biological systems, depending on corona properties, and how these modulate biological uptake. Pristine NM sizes are useful to characterize the initial average sizes and size distributions of NMs but we need to become better at predicting the change in NM size and surface composition in different environments. Lack of knowledge of the dynamic changes that occur when NMs are in biological or environmental compartments blunts our ability to understand and predict how NMs are taken up by cells. As the characterization of corona composition and its evolution in biological systems improve, we will gain increasing confidence in predicting the 'biological relevant entity' that ultimately affects the biological responses to NMs. Once the methods and procedures for NM testing in appropriate environments are developed and fully validated, they need to be converted into regulatory-relevant, practical recommendations.

### 2.4. Ambiguity 4: regulating NMs

Nanotechnology was at an early stage of development when the EU's Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) Regulations came into force in 2007 (Commission 2006). These regulations aimed to ensure safe production, use, and import of substances. The developments in nanotechnology triggered a need for modification of the EU's existing chemical legislation to cover nanoscale forms of materials. This was partly addressed by amending the REACH Regulation annexes (Reach 2018) and corresponding ECHA guidance. However, these changes have been based on knowledge from relatively simple NMs (e.g. metal oxides and carbon-based materials) that are smaller versions of familiar bulk substances. The capability of updated REACH annexes and guidance documents to estimate and manage potential impacts of more complex, functional NMs already in medical use remains to be tested.

These four ambiguities (i.e. definitions, categorization, standardization, and regulation) add to the existing complexity in nano-environmental, health, and safety (EHS) issues. First, difficulties in finding a universally agreed definition and classification of a NM differentiated from its bulk correspondent present serious challenges for the nano-safety research and the safe use of NMs outside the research environment. Second, identifying the most important NM properties and functions contributing to their toxicity is only possible with the availability of reliable and extensive characterization data; this is currently limited by methodological complexities. Lastly, the uncertainties about regulatory requirements for NMs have direct impact on selecting relevant toxicity endpoints for risk assessment and judging the acceptability of measured risks on the basis of risk-benefit considerations for each NM. Resolving these ambiguities by generating new data, developing new tools to learn from the data, and discovering new ways of interpreting the data would directly benefit nanosafety research in multiple ways. For example, the ability to group NMs based on structural/physicochemical similarity would enable regulators to focus their limited testing resources on NMs of high toxicity concern, and to fill data gaps without requiring additional time and cost-intensive animal studies. Moreover, having clear frameworks and guidelines detailing what qualifies as NM and what properties/endpoints need to be tested as part of regulatory risk assessment would help incorporate safety into the design stage and ensure regulatory clarity that improves compliance. In order to resolve remaining ambiguities in nanoscience, it is essential to establish an international network of scientists with multi-disciplinary expertise, policymakers, and industry leaders fully committed to ensuring safer nanotechnology and nano-enabled products.

## 3. Regulatory hazard assessment of NMs

It is now generally agreed that (Donaldson and Poland 2013; Krug 2014) nanotoxicity is not as *specific* as it was first thought to be, so it is unlikely

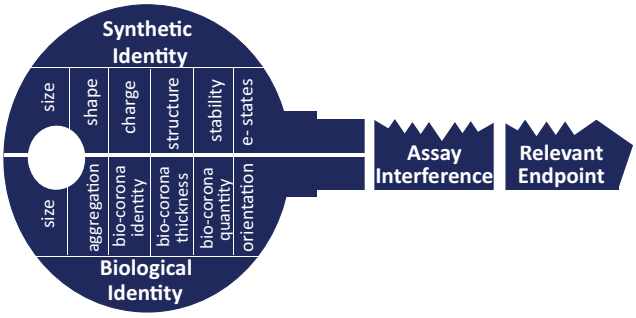


**Figure 2.** The key factors to consider for hazard assessment of NMs.

that completely new risk assessment protocols will be required. However, there are additional issues that apply specifically to NMs, such as interference with toxicity assays (Guadagnini et al. 2015) and formation of the protein corona (Docter et al. 2015; Walkey and Chan 2012). Questions like 'which tests are reliable for identifying potential health effects of NMs' and 'how to translate the acquired knowledge into a regulatory context' need to be clarified in order to avoid false positives or negatives and misinterpretation of toxicity data in nano-safety research. Key steps to consider for hazard assessment of NMs are summarized in Figure 2.

The paucity of faster methods of synthesis and characterization means that we are exploring a minute fraction of possible NMs. This, in turn, means that data that could be used to train ML models of NM structure–activity and structure–property relationships are sparse. The models are therefore less predictive and even those that perform well have small domains of applicability, limiting their use to leverage existing experimental data into new regions of NM space. Clearly, expanding the scale of synthesis and characterization will provide greater insight into the properties of NM that can be used to design improvements, and will have the added benefit of substantially improving the predictivity and applicability of ML models of NM properties. If these models are more predictive and more widely applicable, it makes possible more rational 'safe-by-design' NMs.

The inability to predict *in vivo* impacts of NMs is largely due to the cost and ethical limitations of animal testing, and the relatively poor correlations between *in vitro* assessments of the biological effects of NMs and their *in vivo* effects. Use of organ-specific cell lines derived by regenerative medicine techniques and a better understanding of how NM impacts on biological systems as assessed by omics technologies inform toxicity mechanisms, and allow *in vivo* effects of NMs to be more accurately predicted without substantially increasing animal testing. All of these developments will provide much better tools for regulatory agencies to assess or even predict the likely risk and hazard of new NMs, allowing appropriate regulation.

### *3.1. Understanding the physicochemical identity of NMs*

A thorough understanding of the *physicochemical* and the *biologically-relevant entities* is critical for linking biological activity to intrinsic materials properties, and to allow toxicity to be predicted for untested materials using these structure–activity relationships (Farrera and Fadeel 2015). This knowledge can be also be used to reduce the toxicity of substances through structural modifications and to *design-out* hazards without compromising performance (so-called safety-by-design) (Yan et al. 2019). In addition to designing out toxicity during the development of new NMs, the knowledge of toxicity-driven nano-scale properties would further assist in understanding the mechanisms by which NMs interact with biological systems and prioritizing which NMs should be subject to extensive experimental investigation.

There is still no scientific consensus on the minimum set of relevant characteristics for toxicological evaluation (Krug 2018; Kar, Ghosh, and Leszczynskia 2019; Gajewicz and Puzyn 2019). The key physicochemical features considered important in the majority of cases (Sayes and Warheit 2009; Powers et al. 2007; Boverhof and David 2010) include: morphological characteristics (particle size, shape, and their distribution); surface characteristics (chemistry, charge, and modifications); solubility; and colloidal stability and state of agglomeration. Numerous studies in recent years have shown that NMs may display size-dependent (Wongrakpanich et al. 2016; Gliga et al. 2014; Pan et al. 2007; Napierska et al. 2009), shape-dependent (DI Bucchianico et al. 2013; Zhang et al. 2017; Niu et al. 2016; Woźniak et al. 2017) and surface-dependent (Yang et al. 2012; Hoshino et al. 2004; EL Badawy et al. 2011) toxicity.

Table 1 lists the key toxicity-related physicochemical parameters of NMs.

The key considerations when characterizing NMs prior to toxicological evaluation are:

1. Measuring not only 'as-received' intrinsic properties but also properties in relevant media,
2. Quantifying a single characteristic over an extended period of time using multiple techniques, especially when a priori knowledge on the parameter of interest is unavailable for the test material,
3. Providing detailed information (metadata) on measurement conditions, such as sample preparation, pH value, and concentration (Warheit 2008).

**Table 1.** The key toxicity-related physical, chemical and behavioral parameters of NMs.

| Property type | Key property |
|---|---|
| Physical properties | Particle size (mean and distribution) |
| | Particle shape (dimensions and aspect ratio) |
| | Specific surface area |
| | Density |
| | Porosity |
| | Roughness |
| | Viscosity |
| Chemical properties | Composition (core, surface, overall) |
| | Surface properties (charge, coating, affinity) |
| | Functionalization |
| | Purity/impurities |
| | Chemical structure |
| | Crystallinity/defects |
| | Redox activity |
| Behavioral properties | Solubility |
| | Dispersibility |
| | Corrosivity |
| | Dissolution rate |
| | Degradation rate |
| | Dustiness |
| | Hydrophobicity |
| | Surface reactivity |
| | Aggregation/agglomeration |

### 3.2. Understanding the biologically relevant NM identity

In biological fluids, the surfaces of NMs are immediately coated by a layer of adsorbed proteins (the protein corona) and ions. These materials have high affinity for biomolecules and ions resulting in their physicochemical identity being transformed into a biological one (the biologically relevant entity). This is a dynamic process in which the composition of the corona changes in different biological fluids, and over time as more abundant lower affinity proteins are replaced by less abundant higher affinity proteins. Since the toxic potential of NMs depends on their size and surface characteristics (He et al. 2010; Greish et al. 2012; EL Badawy et al. 2011), the risk they pose may also change accordingly when they are aggregated or coated with other molecules in biological environments. Moreover, biological entities such as cells interact with the entire NM-corona complex (Figure 3), not just with the core NM. Therefore, it is critically important to investigate protein corona formation and its structure prior to toxicity testing (Docter et al. 2015). Such knowledge may help to understand the true correlation between structural features and biological

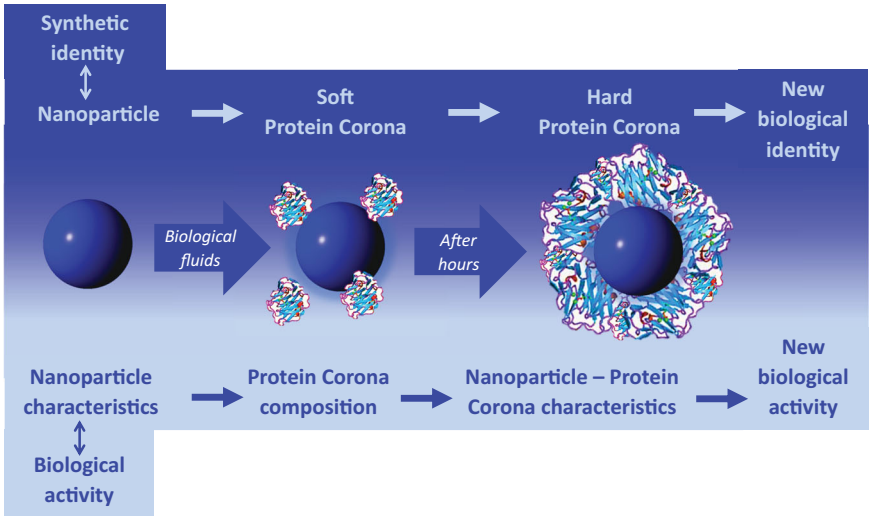


**Figure 3.** Nanoparticle-protein corona complex.

effects and explain some of the inconsistencies in *in vitro* and *in vivo* studies.

Currently, the main concern in nanotechnology-related EHS research is not only to identify which physicochemical or biological properties are responsible for toxicological effects but also to link hazard with toxicity-related features in a quantitative way. Altering the biological activity by modifying toxicity-related properties is only possible if the relationship between physicochemical characteristics, toxicity, and the desired functionality is mathematically defined. The field of computational nanotoxicology has emerged to meet this need but it is challenged by lack of sizeable and consistent datasets, the complexity of nanostructures, and a need for more multidisciplinary-trained researchers in this new field. More data on *in vitro* and *in vivo* effects of well-characterized NMs are needed for data-driven methods to reach their full potential and to fully decode the relationship between physicochemical structure and biological activity.

### 3.3. Understanding the main entry routes of NMs

NPs may enter the human body by inhalation, ingestion, or skin contact, and travel in the bloodstream to internal organs where they can cause harm. The main routes by which NPs can enter the body are shown in Figure 4.

It is now well known that the majority of non-targeted NPs tend to accumulate in the liver or spleen (Tsoi et al. 2016). Most preliminary studies have shown that a large fraction of uncoated NPs that are distributed to major organs such as liver are cleared by the immune system within a short period of time (Choi et al. 2010). However, accumulation in secondary organs following long-term exposures and the biological mechanism by which NPs are immunologically sequestered from the body need further investigation. While no vital danger has been proven, scientific evidence so far provides incomplete picture of the organ distribution and clearance of NPs (and their agglomerates) from the body (Buckley et al. 2017). Such understanding is important for not only predicting the potential toxicological implications of accumulated NPs in human tissues and organs, but also controlling the biodistribution of NPs with the ultimate aim of targeting unhealthy cells (e.g. tumors) while leaving the healthy ones intact (Haute and Berlin 2017).

It is apparent from previous scientific studies that a clear link exists between synthetic identity of NMs, their bioactive interface within a biological system, and the level of cellular internalization and

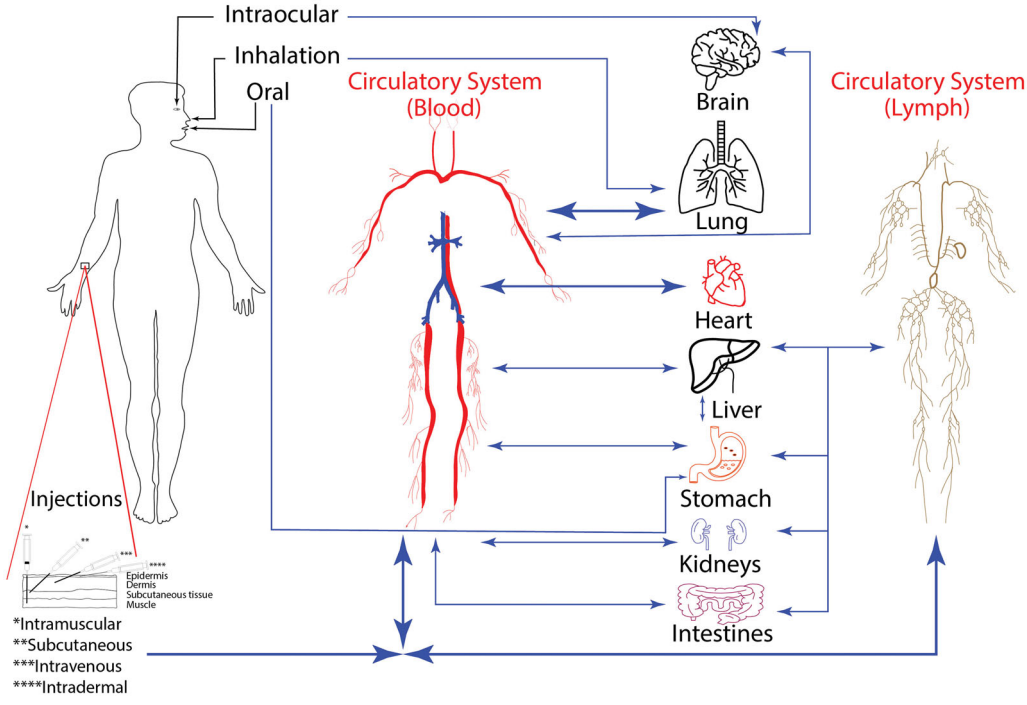


**Figure 4.** Potential entry routes and distribution of nanoparticles in the body.

biodistribution. For example, the intentionally designed physicochemical properties of NMs (e.g. particle size, shape, surface chemistry, and functionalization) change as a function of biological environments, and these changes are reflected in how NMs are seen by the cellular systems. Therefore, analyzing these interrelated concepts in a manner meant to explain the multi-way relationships among them is critically important to arrive at any conclusion regarding the entry of NMs into the body and the underlying parameters controlling these cellular interactions.

### 3.4. Understanding how NMs affect testing systems

Reliability of existing *in vitro* approaches for the assessment of NMs health hazard potential has been a subject of continued debate for the past two decades. Although the question of 'which tests can be safely used to assess the hazard of NMs' continues to arouse controversy, expert consensus favors the use of testing systems with minimum interference potential (e.g. interference of NMs with nanotoxicology assays or assay reagents) to avoid under- or over-estimation of toxicity.

An important technical limitation of conventional assays is caused by putative interference between NMs and the assay system (Guadagnini et al. 2015). While assay interference is not a new phenomenon, specific properties of NMs (e.g. increased surface area, catalytic activity, optical and magnetic characteristics) can interfere with assays that rely on changes in absorbance or fluorescence to provide information on cellular activities. Several recent studies have exemplified NM interference with *in vitro* systems (Ahmed et al. 2017; Wang et al. 2012; Geys, Nemery, and Hoet 2010), generating both false-positive or false-negative results. For example, NMs have been shown to absorb analytes, react with assay components, release chemical species, and cause side reactions (Ong et al. 2014). Therefore, systematic evaluation of possible NM-assay interferences under realistic conditions is essential to ensure valid interpretation of test results. This will lead to necessary protocol modifications and nano-specific interference controls. Assay-specific interferences and possible solutions are summarized in Table 2. As most of the traditional *in vitro* methods exist for the identification of toxicological hazards have not been specifically validated for each NM class, possible interferences and solutions given in Table 2 are more general in nature and not specific to any NM.

The ways in which potential assay interferences depend on particular physicochemical properties, working conditions of the assay, and the loading/exposure protocols applied make drawing general conclusions about the reliability of certain tests for all NMs very difficult. With so many factors

**Table 2.** Assay-specific interferences and possible solutions.

| Assay | Test | Potential interference | Potential solution |
|---|---|---|---|
| Cell viability | 1. LDH | • Optical interference<br>• Inactivation/adsorption of LDH | • Use lower concentrations<br>• Use cell-free controls |
| | 2. Neutral red | • Dye adsorption<br>• Interference with readout system | • Use lower concentrations<br>• Intensive washing steps |
| | 3. Annexin V | • Interaction with serum proteins | • Confirm with other assays<br>• Use spike-in controls |
| | 4. ATP | • Optical interference | • Avoid light emitting NMs |
| | 5. TUNEL | • Nonapoptotic DNA cleavage | • Confirm with other assays<br>• Digest proteins |
| Metabolic activity | 1. MTT<br>2. MTS<br>3. XTT<br>4. WST1 | • Optical quenching/interference<br>• Interaction with formazan salts, serum proteins, or dye | • Use lower concentrations<br>• Pre/post-spike controls<br>• Centrifugation<br>• Use modified salts<br>• Adapt cells to serum-free medium |
| Oxidative stress | 1. EPR<br>2. ESR | • Interaction with paramagnetic molecules | • Use stable probes |
| | 3. $H_2$DCF-DA | • Optical quenching/interference | • Thoroughly wash samples<br>• Confirm with other assays |
| Inflammation | 1. ELISA | • Interaction with Interleukins-cytokines | • Careful design<br>• Pretest using cell-free media |
| Genotoxicity | 1. COMET | • DNA fragmentation | • Confirm with other assays<br>• Use lower concentrations<br>• Prevent agglomeration |
| | 2. Micronucleus | • Interference of cytochalasin-B | • Careful design (serum, exposure time/order) |

contributing to assay interference, a paucity of knowledge on possible interference mechanisms, and the fact that NMs exhibit novel physicochemical properties, confidence in the results of toxicity testing can only be achieved by validating each assay for each NM formulation, and using complementary assays for common endpoints, especially if doubt exists. It is also advisable to use appropriate controls, realistic concentrations, and maintain a high level of suspicion when inspecting test results so as to detect and control interferences that may lead to erroneously high or low results.

### 3.5. Testing nano-hazards

Conventional toxicity assessment relies primarily on animal testing that is very costly, slow, and ethically problematic. With the rapid development of new materials and strong growth in existing technologies (e.g. biotechnologies), the need for faster and cheaper non-animal test methods for regulatory applications has become urgent.

The term non-animal testing in the context of hazard assessment refers to the use of human cells/tissues (*in vitro*) and computer-modeling (*in silico*) methods as alternatives to *in vivo* animal testing. *In vitro* approaches are employed worldwide to detect adverse effects of NMs such as cytotoxicity (Brunner et al. 2006; Hu et al. 2009), immunotoxicity (Dobrovolskaia, Germolec, and Weaver 2009; Smith et al. 2014), and genotoxicity (Gonzalez, Sanderson, and Kirsch-Volders 2011; Doak et al. 2012; Qiao, An, and Ma 2013). In particular, the ability of NMs to trigger oxidative stress in biological systems is the most frequently reported cause of nanotoxicity (Manke, Wang, and Rojanasakul 2013; Lehman et al. 2016). However, the oxidative potential should be seen as a toxicological parameter rather than the main mechanism of nanotoxicity as the observed link could be a *consequence* of NM-induced toxicity, not necessarily the *cause* (Kodali and Thrall 2015). While conventional *in vitro* assays are an important first step toward assessing the potential risks of NMs, there is a need to establish fully validated test systems and procedures to bring old practices in line with the products of new technologies. In particular, the correlation between *in vitro* and *in vivo* responses needs to be made more robust if *in vitro* methods are to be used as viable surrogate assays to replace animal testing.

*In silico* approaches make much better use of the available experimental data on hazard, allowing new knowledge to be extracted that can be used to 'design in' safety for new materials without compromising desired functionality (Oksel et al. 2015). Clearly, most non-testing approaches are data-driven, requiring experimental information to train them and cannot (yet) completely replace animal testing in toxicology. However, these methods are capable of making maximum use of often scarce and expensive experimental data, providing insights into toxicity mechanisms, filling data gaps, prioritizing potentially problematic materials for testing, and reducing animal testing by eliminating non-critical experimental processes. The current state of research on the use of *in silico* methods and issues still to be addressed, are summarized in a recent review (Winkler 2020). Although there is a profound interest among policy-makers and the scientific community to move from animal-based individual toxicity assessments toward a more integrated hazard screening approach, the lack of practical guidance on the harmonized use of non-animal testing methods in regulatory context has resulted in low regulatory and industrial acceptance so far (Tantra et al. 2015). The key to the successful uptake of alternative methods by scientists and regulators is to transparently demonstrate the reliability and relevance of their outcomes for hazard screening and assessment purposes.

### 3.6. Using realistic concentrations and dose metrics

The basic concept of toxicology, *the dose makes the poison*, has not been fully adopted in the field of nano-safety (Lison, Vietti, and Van De Brule 2014). Selection of realistic exposure concentrations and physiologically relevant measures of dose is needed (and currently lacking) for meaningful comparison of *in vitro* outcomes with previously published *in vitro* data and *in vivo* biological responses (Cohen, Deloid, and Demokritou 2015). Unlike conventional materials whose toxic doses can solely be described by administered mass or concentration, NMs requires a careful adaptation of traditional dose-metrics as mass alone is often not sufficient to

describe their property-dependent dose–response relationship (Delmaar et al. 2015). Earlier *in vitro* nanotoxicity studies have reported studied doses in mass units (μg/mL), ignoring surface- or number-related effects (Deloid et al. 2015). With the recognition of the need to move beyond mass-only metrics for NMs, various dose-metrics such as particle number, volume, surface area, and body burden have been suggested, each with some limitations. In the absence of universally agreed dose measures that can adequately reflect NM exposure, reporting concentrations in a range of dose metrics will allow for different interpretations of exposure. Special attention should be given to NM dispersion preparation and characterization to ensure accurate dosimetry and delivered to cell doses of particles (Cohen, Deloid, and Demokritou 2015).

### 3.7. Translating knowledge into regulatory outcomes

Generating reliable nano-hazard data is one issue but translating these pre-normative research results into regulatory outcomes is an entirely different problem. In general, regulators' early concerns about lack of nanotoxicity data have been replaced by lack of regulatory-relevant data. Although large volumes of nanotoxicity data have been generated in the last two decades (Oksel, Ma, and Wang 2015), the vast majority of these data suffer from consistency problems between replicate samples, methods, analysts, or laboratories (Furxhi et al. 2020; Robinson et al. 2016). Much of this provides information of NM *hazard*, while modeling of the resulting *risk* when NMs are used in diverse workplaces and exposure scenarios, is less well developed. In the absence of reliable and consistent data needed to broaden the scope of existing laws to cover nano-specific issues, regulators take a precautionary approach or use the best available evidence to regulate NMs. However, overly cautious measures that are disproportionate to the real risk may stifle innovation, progress in the field of nanotechnology, and commercial applications. On the other hand, failing to properly address possible risks from nano-enabled products may have severe effects on public health and the environment, resulting in a backlash against NMs. The main risk management challenge under considerable uncertainty is to find the right balance between real risk and benefit.

Newly acquired information can only be applied to regulatory tasks if the key policymakers and legislators are able to translate, interpret, and extrapolate it. Therefore, the key to the successful integration of new information into regulatory frameworks and decision-making processes is to transparently demonstrate the reliability and relevance of their outcomes for regulatory purposes. To facilitate the flow of information from production to policy use, following barriers need to be addressed:

1. providing an easy access to data,
2. generating verifiable, consistent, and high-quality data,
3. fostering interdisciplinary and collaborative research,
4. developing working relationships between policy-making bodies, regulatory authorities, and other relevant stakeholders, and
5. increasing openness of regulatory bodies to new information and tools.

### 3.8. Comparison with current FDA/EMA regulations and guidance in related fields

The FDA and European Medicines Agency (EMA) have comprehensive regulations and guidance documents for drugs and medical devices, some of which provide insight into how biomedical NMs could be regulated. The FDA has classifications for ~1700 different types of devices and has grouped them into 16 medical specialties (panels). Each type of device is assigned to one of three regulatory classes based on the level of control required to assure device safety and effectiveness:

1. **Class I General Controls** e.g. nasal oxygen cannulas, manual stethoscopes, and hand splints represent a low risk to the patient;
2. **Class II General Controls and Special Controls** e.g. tracheal tubes, bone plates, elbow joint radial prostheses. These are typically surgically implanted into the body or by some other medical intervention and represent a moderate risk to the patient.
3. **Class III General Controls and Premarket Approval** e.g. aortic valves, constrained metal

**Table 3.** A simplified summary of medical device classification and regulation in the EU and US and possible implications for NM regulation.

| EU classifications and regulatory requirements | | US classifications and regulatory requirements | | Possible implications for NM classifications and regulatory requirements | |
|---|---|---|---|---|---|
| *Class I* | Declaration of conformity by manufacturer, registration of product | *Class I, largely external, low risk to patient* | Most devices exempt from FDA notification and regulation, and listing requirements apply | *Class I, low hazard materials, confined in matrices, negligible uptake by humans or environment, low risk* | Exempt from full notification, summary of NM properties and risk profile |
| *Class I measuring or sterile* | Notified Body approval is required to assess the sterility or measuring aspects of the device | | | | |
| *Class IIA* | Conformity assessment by a Notified Body (QMS, TD for device category, PQA, PV, DoC) | *Class II, surgically or otherwise implanted, medium risk to patient.* | 510(k) application for new/modified devices to demonstrate substantial equivalence to a predicate device. If not equivalent, PMA application | *Class II, medium or high hazard NMs in inert matrix, some potential for uptake during manufacture or disposal, medium risk* | Simplified registration as a new chemical under REACH/CLP and US EPA |
| *Class IIB* | Conformity assessment by a Notified Body (QMS, TE, TD for generic device group, PQA, PV, DoC) | | | | |
| *Class III* | Conformity assessment by a Notified Body (QMS, TE, TD for every device, PQA, PV, Consultation, DoC) | *Class III, surgically implanted, high risk to patient* | PMA application to provide scientific evidence to support safety/efficacy, unless pre-amendment Class III device | *Class III, high hazard NMs, or medium risk used internally in man, high risk* | Full registration as a new chemical under REACH/CLP and US FDA/EPA |

QMS: quality management system; TD: technical documentation; PQA: production quality assurance; PV: production verification; DoC: declaration of conformity; TE: type examination; FDA: Food and Drug Administration; PMA: pre-market approval; REACH: Registration, Evaluation, Authorization, and Restriction of Chemicals; CLP: classification, labeling, and packaging; EPA: Environmental Protection Agency.

hip prostheses, and coronary stents with the highest patient risk.

The assigned class determines what premarketing submission/application is needed for FDA clearance to market. If your device is classified as Class I or II, and if it is not exempt, a 510 k premarket notification is required.

The EMA regulates new drugs and medical devices. It evaluates the quality, safety, and efficacy of marketing authorization applications for drugs, medical devices, and medical devices that also incorporate a medicinal product. Table 3 shows, medical device classification and regulation in the US and EU are similar. The way these are regulated into three main classes provides possible guidance for streamlined regulation of NMs, as is suggested in Table 3.

In the EU, NMs are defined as any other substances under both existing REACH and classification, labeling, and packaging (CLP) regulations. An EU definition of NMs is used to help harmonize how NMs are defined across REACH and CLP legal frameworks. Specific REACH legal requirements apply to companies that manufacture or import nanoforms: characterization of nanoforms or sets of nanoforms covered by the registration (Annex VI); chemical safety assessment (Annex I); registration information requirements (Annexes III and VII-XI); and downstream user obligations (Annex XII). Since REACH and CLP cover NMs, ECHA must carry out its tasks for nanoforms within the various REACH and CLP processes as it would for any other form of a substance.

Miernicki et al. recently discussed the issues involved in regulation of NMs from an EU perspective (Miernicki et al. 2019). They made the following recommendations for the regulation of NMs that would benefit not only European law, but other jurisdictions in which legal approaches to NMs are considered:

1. NM definitions should be clarified by avoiding ill-defined terms and by including clear thresholds (e.g. for solubility in the Cosmetics Regulation) for the sake of legal certainty and workability of the regulations.
2. Nano-specific regulations that are not workable in practice cannot fulfill their function, e.g. to

protect humans and the environment, and thus need adaptation.
3. Adaptation clauses should be harmonized and include clearer distinction between technical/scientific aspects to be adapted by the Commission and political/risk management aspects that should remain within the responsibility of the legislator.
4. Product manufacturers should carry the burden of proof for the NMs' origin.
5. The 50% by number threshold should be replaced by a threshold of 1% by weight to make definitions workable with current particle analysis methods, contributing to a more balanced cost–benefit relation in the regulatory nano-framework and its enforcement.

## 4. Biomedical applications of NMs

Rapid developments in (bio)medical research and technology have contributed to increasing human life expectancy, which has resulted in an increase in the number of ageing patients requiring medical care (Robertson and Williams 2009). NMs can play important role in early diagnosis and treatment of serious illnesses such as cancer. A short summary of biomedical technologies employing NPs are summarized below. Interested readers are referred to recent, comprehensive reviews in this field (Mohammed et al. 2017; Arias et al. 2018; Burdușel et al. 2018; Elahi, Kamali, and Baghersad 2018; Han et al. 2019; Maiti et al. 2019; Alcaraz et al. 2020; Makvandi et al. 2020; Park et al. 2020).

### 4.1. Contrast enhancing agents in biomedical imaging

NM selective accumulation in tumors, and their ease of functionalization, make them important contrast enhancing agents in biomedical imaging (Nakamura et al. 2016). Dipeptide NPs (Fan et al. 2016), semiconductor quantum dots (Gao et al. 2004), thermosensitive fluorescent rhodamine 6 G NPs (Cheng et al. 2017b), pyrene loaded supramolecular micelles (Cheng et al. 2017a), conjugated NPs (Pecher et al. 2010), and functionalized fluorescent dyes (PEGylated C18-R) (Zhang et al. 2014) have demonstrated enhanced emission, reduced nonspecific binding, and better *in situ* stability (Caponetti et al. 2019). Targeted paramagnetic NMs (Winter et al. 2003), superparamagnetic iron oxide NPs (SPION) (Zhang et al. 2009), pH-sensitive calcium phosphate-PEG shell NPs (Mi et al. 2016), SPION loaded red blood cells (Rahmer et al. 2013), DNA plasmid loaded SPIONs (Park et al. 2008), and fluorinated graphene oxide NPs (Romero-Aburto et al. 2013) are recently developed MRI contrast agents with favorable superparamagnetic characteristics, biocompatibility, and ease of modification. Gold NPs are also important contrast agents for CT imaging due to their unique optical properties, high x-ray attenuation, low toxicity, and ease of surface functionalization (Yu, Yang, and Sun 2020; Chhour et al. 2016; Keshavarz et al. 2018; Lin et al. 2017; Kim et al. 2007; Peng et al. 2012). $^{18}$F-, $^{64}$Cu-, $^{199}$Au-, and $^{111}$In-labelled NMs have been developed for PET and SPECT imaging (Devaraj et al. 2009; Glaus et al. 2010; Zhao et al. 2016; Sampath et al. 2010). NMs developed for biomedical imaging have superior performance to conventional agents, but few have been translated to the clinic (Thakor et al. 2016).

### 4.2. Antimicrobial agents

Some NPs exhibit high antimicrobial activity useful for treating surgical wound infections. Silver NMs accelerate wound healing (Gunasekaran, Nigusse, and Dhanaraju 2011) and fight post-surgical infections (Santos et al. 2019) due to broad-spectrum antimicrobial activity. Titanium-doped silver NPs prevent multidrug-resistant infections (Cochis et al. 2016) while silver NP embedded titania nanotubes exhibit persistent antibacterial effect against pathogenic *Escherichia coli* and *Staphylococcus aureus* (Gao et al. 2014). Nanoscale silver coatings are effective against implant-associated infections (Kuehl et al. 2016). Copper, titanium, gold, and zinc NPs have broad-spectrum antimicrobial activities due to induction of oxidative stress (Khezerlou et al. 2018).

### 4.3. Therapeutic NMs

Magnetic NMs are increasingly used for treatment of diseases, especially cancers. Magnetic NM clusters and colloidal crystals (nanobeads) have diameters 50–200 nm. They are very useful for tissue targeting,

tissue ablation, and imaging (Winkler 2017). Heating due to hysteresis losses, which occurs when a fluid containing magnetic NMs is exposed to an alternating external magnetic field, can selectively damage tumors. It is particularly useful for hard to treat cancers like hepatocellular carcinomas.

The efficiency of traditional cancer drugs is often hampered by biological transport barriers, leading to therapeutically insufficient drug concentrations at the target site (e.g. tumors). Due to their high degree of flexibility in design, drug-loaded NPs can be modified so that they bind to the target diseased cells (and not to neighboring healthy cells), pass through the surface, get carried inside, and release the drug when they get to the center of target cell. NPs that are loaded with cancer drugs provide an alternative way of diagnosing and attacking aggressive cancers without the side effects of traditional treatment methods. In the future, researchers hope to design intelligent bionanorobots that can look for signs of distress in the bloodstream, detect diseased cells at their earlier development stages, and force them to self-destruct without harming surrounding tissue.

### 4.4. Tissue engineering

Tissue engineering research aims to develop biological constructs for repair, restoration, maintenance, or improvement of tissue function (Buchholz 2007). Second generation biomaterials with biological and mechanical properties more similar to those of human tissues have evolved from first-generation biological substitutes (Fahey and Indelicato 1994). Biomaterials can trigger immune responses because they do not mimic the highly complex extracellular matrix, leading to rejection of implanted materials (Clark, Ghosh, and Tonnesen 2007; Hynes 2009). Tissue engineering at the nanoscale allows design of new biologically-inspired materials with properties that overcome the limitations of conventional tissue engineering materials (Holmes et al. 2016; Fathi-Achachelouei et al. 2019; Fleischer and Dvir 2013). A wide range of nanoscale biomaterials, including inorganic, ceramic, polymeric, and metallic NPs, have been employed in tissue engineering applications, such as enhancement of cell proliferation rates, novel mechanical and electrical properties of scaffolds, gene deliver, and fabrication of 3-D tissue-engineered constructs (Hasan et al. 2018). For example, nanostructured calcium phosphates and nano-hydroxyapatite are used as bone substitutes due to their biocompatibility, osteoconductive properties, and bone regenerative capacity (Chen et al. 2012; Barba et al. 2018). Similarly, nano-scale bioprinting of 3-D hydrogel scaffolds is an active area of research with enormous potential to resemble natural bone tissue and the cells' natural surrounding environment (Markstedt et al. 2015; You et al. 2018).

### 4.5. Biosensors

Biosensors use biomolecules, tissues, and organisms to measure concentrations of specific biological analytes, a biological structure, or a microorganism (Lafleur and Yager 2013). They convert a molecular recognition event into a signal (e.g. optical, electrical, or magnetic) that provides information about health and diseases, enabling earlier disease detection and more targeted therapies (Mohanty and Kougianos 2006). The small size and large surface-to-volume ratio of NMs make them well suited for medical biosensing applications where enhanced sensitivity and detection capability are essential. Nanostructured carbon materials e.g. nanotubes with high sensitivity and extremely low detection limits (Sireesha et al. 2018), have been used in biosensing applications for over two decades (Balasubramanian and Burghard 2006). Their electronic/optical properties and permeability through biological membranes make them well suited to minimally-invasive, *in vivo* optical biosensing applications (Hofferber, Stapleton, and Iverson 2020). Quantum dots are widely used in fluorescence-based medical biosensors (Zhu, Li, and Cheng 2013). Other nanobiosensors include gold nanorod- and graphene oxide-based electrochemical biosensors for early detection of cancer (Azimzadeh et al. 2016), inorganic nanocrystal-based sandwich immunoassays for multitarget detection of proteins (Liu et al. 2004), nanosized silica-based immunosensors for prostate cancer detection (Qu et al. 2008), and nanosilver-based plasmonic biosensing applications (Loiseau et al. 2019). As the scientific evidence for the benefits of nanobiosensors grows, so too have concerns about their *in vitro* and *in vivo* biosafety. Clinical translation of these systems hinges on

understanding how the human body responds to, distributes, and eliminates biomedical NMs, with the ultimate aim of ensuring their safe use in biosensing applications.

## 5. Safety of biomedical NMs

With the ever-increasing use of nanostructures in biomedical applications, human, and environmental exposure to NMs has become inevitable. There are existing and robust regulatory processes in place for biomedical NMs used for diagnostic applications. There is also strict regulation of implantable and indwelling medical devices that increasingly contain nanostructured coatings. For example, the US FDA defines three risk classes for medical devices and devices that are not within a type marketed before are automatically classified into class III (high risk), a cautious approach that is suitable for NMs with unconventional properties. Review and approval of nanoscale drugs, coatings, and devices are ongoing (Paradise 2019), e.g. the FDA has approved nanoformulations of paclitaxel and doxorubicin as new cancer drugs, of the immunosuppressant sirolimus, and of an estradiol topical emulsion (Wagner et al. 2006). Regulation will need to be agile to deal with new technologies such as the use of microscale and nanoscale topographies to control biological responses such as microbial pathogen attachment, and modulation of immune responses by novel coatings (Vassey et al. 2020).

## 6. Final remarks

Advances in systems biology, chemistry, automation, and computer science have led to several paradigm shifts in regulatory safety assessment. These include use of animal data for estimating health impacts of chemicals on humans and the environment, development of faster and cheaper non-animal alternatives to animal tests, use of gene expression and other omics data, faster high capacity *in vitro* screens, and robust *in silico* methods. The ultimate aim is to accelerate safe manufacturing and use of products, while reducing costs and the time from design to commercialization. Despite the growing interest among regulatory authorities in the development of time- and cost-effective methods to complement and extend traditional risk assessment methods, there are significant barriers to integrating such concepts into the practice of existing regulatory frameworks.

The safety evaluation of biomedical NMs requires input from multiple sources and disciplines. The successful adaptation of risk assessment procedures to NMs directly depends on the ability of experts in material science, toxicology, industry, and regulatory bodies to understand how their respective expertise complements that of the others. There is a clear recognition of the value of such cross-disciplinary collaboration for improving chemical risk assessment processes. However, only a few ideas have been reduced to practice so far, as scientists, regulators, and industry work from different assumptions and are invested in their own points of view.

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