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Nanomedicines - Tiny particles and big challenges

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ABSTRACT

After decades of research, nanotechnology has been used in a broad array of biomedical products including medical devices, drug products, drug substances, and pharmaceutical-grade excipients. But like many great achievements in science, there is a fine balance between the risks and opportunities of this new technology. Some materials and surface structures in the nanosize range can exert unexpected toxicities and merit a more detailed safety assessment. Regulatory agencies such as the United States Food and Drug Administration or the European Medicines Agency have started dealing with the potential risks posed by nanomaterials. Considering that a thorough characterization is one of the key aspects of controlling such risks this review presents the regulatory background of nanosafety assessment and provides some practical advice on how to characterize nanomaterials and drug formulations. Further, the challenges of how to maintain and monitor pharmaceutical quality through a highly complex production processes will be discussed.

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Abbreviations: ANMAT, Argentinian National Administration for Food Drugs and Medical Technology; ANVISA, Brazlian Health Surveillance Agency; API, Active pharmaceutical ingredient; CDER, Center for Drug Evaluation and Research; CDSCO, Central Drug Standard Control Organization; CS-FDA, Chinese State Food and Drug Administration; DLS, Dynamic light scattering; EC, European Commission; ECHA, European Chemicals Agency; EEA, European Economic Area; EFSA, European Food Safety Authority; EMA, European Medicines Agency; EU, European Union; EUON, European Union Observatory for Nanomaterials; GB, Guobiao (national standard); GMP, good manufacturing practice; ISO, International Standardization Organization; IUCLID, International Uniform ChemicaL Information Database; NBCD, non-biological complex drug; NTA, Nanoparticle Tracking Analysis; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; SAC, Standards Administration of China; SCENIHR, Scientific Committee on Newly Identifed Health Risks; TSCA, Toxic Substances Control Act; USA, United States of America; US-EPA, Environmental Protection Agency of the United States; US-FDA, Food and Drug Administration of the United States; USD, United States Dollar; VSSA, volume specific surface area.

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1. Introduction

Today, the global nanomedicine market covers a wide spectrum of interventions including nanostructures for the diagnosis, prevention, or treatment of diseases. Recent estimates project a compound annual growth rate of 12.6% reaching a total value 261 billion USD by 2023 [1]. And with a growing influx of nanotechnology-related products into the national markets, regulatory authorities all over the world have become aware of the potential risks and hazards nanomaterials may pose to human health and the environment [2].

Many nanomaterials employed by the pharmaceutical industry fall under the national legislation on chemicals before they are further processed to pharmaceutical products or medical devices. Commonly, substances or final products with one or more dimensions in a size range between 1 and 100 nm, are considered nanomaterials [4,5]. In such cases, manufacture, controls, distribution and labeling can underly specific requirements [6–14]. The safety assessment of chemicals takes aspects of occupational health and environmental toxicity into consideration which makes it rather complex to define relevant characterization methods and assays.

At present, the world's largest economies, the United States of America (USA), China and the EU, are setting global standards in the definition (see Table 1), characterization and safety assessment of nanomaterials.

For example, the Environmental Protection Agency of the United States (US-EPA) established a relational database to analyze the effects of nanomaterial emissions on the environment using peer reviewed publications [17]. Similar efforts have been made by other countries [18]. In June 2017, the European Chemicals Agency (ECHA) together with the European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) launched the first phase of the European Union Observatory for Nanomaterials (EUON), a platform to inform workers, consumers but also professionals about nanomaterials in consumer products [19,20].

Once a drug product or medical device exhibits a characteristic therapeutic effect resulting from the use of nanomaterials, a detailed understanding of the extent to which the pharmacodynamic and pharmacokinetic responses have been altered due to the dimensions of the nanomaterial, is required [2,21,22]. EUON's nanotechnology knowledge base includes over 1000 products from various sectors. Under healthcare, a total of 91 products, either drug products or medical devices, have been registered, covering a wide range of indications such as cancer, cardiovascular diseases, diabetes, and infection [20]. They include wound dressings, titanised implants and liposomal drug products (see Table 6 for a list of products containing nanomaterials registered in EU and/or USA). For the US, the Nanomaterial Consumer Products Inventory provides information on 762 nanotechnology-related products in the category fitness and health [23]. However, while the major fraction uses nanosilver (24%), for 49% of the products, there is no further information on the composition of the nanomaterial provided [23].

When focusing on pharmaceutical products, a relatively small number of platform technologies are utilized by the industry as highlighted by a review of submissions to the Center for Drug Evaluation and Research (CDER) of the Food and Drug Administration in the United States of America (US-FDA) (see Fig. 1) [3].

Sometimes classified as non-biological complex drugs (NBCD), lipsomes and some selected nanocrystal formulations have proven to be ambitious to manufacture under the conditions of good manufacturing practice (GMP) and offer many challenges with regards to the physicochemical and biopharmaceutical characterization.

Today, the regulatory framework for nanomaterials impacts the pharmaceutical production chain at all levels, starting with the excipient material and drug substances which are manufactured and processed into drug products or medical devices. In the following, the status quo of a growing regulatory framework and strategies applied to optimize quality and safety of nanotechnology-related products will be discussed in more detail. Some of the most common definitions and terms used by either the scientific community or the regulatory authorities are presented in Table 2. Finally, this review will provide a best practice guide for characterization of nanomaterials according to the current scientific and the regulatory requirements.

2. Status quo of the regulatory framework

As a consequence of the great diversity of nanotechnology, not all biomedical products, diagnostics and devices undergo the time-consuming procedures of regulatory pre-market approval. Starting with the manufacture of raw materials, there are several steps involved in the commercialization of nanomedicines from bench to bedside (see Fig. 2).

For many years, a framework of recommendations and guidelines has been issued to support the industry and also to control the growing nanomedicine market [2]. In the following, an update of the existing rules and regulations will be presented, with a focus on the three most relevant areas:

2.1. Excipients and drug substances

A great variety of pharma-grade excipients, even those being used in conventional products, exhibit structures in the nanosize range. The potential effects of these engineered nanomaterials have been addressed

Table 1
Definitions applied to nanotechnology-related products in the USA [15], China [16] and the EU [4].

*"…a material or end product is **engineered to have** at least one external dimension, or an internal or surface structure, in the nanoscale range (**approximately 1 nm to 100 nm**) or […] a material or end product is **engineered to exhibit properties or phenomena**, including physical or chemical properties or biological effects, that are attributable to its dimension(s), **even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm)**."*	*"Nanomaterials is a material which has a structure in the three-dimensional space in **at least one dimension in the nanometer scale (from 1 nm to 100 nm)** range of geometric dimensions, or constituted by the nano-structure unit **and a material with special properties**"*	*"'Nanomaterial' means a **natural, incidental or manufactured material** containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for **50% or more of the particles in the number size distribution**, one or more external dimensions is in the **size range 1 nm - 100 nm.** In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold **between 1 and 50 %**."*

by the current regulatory framework on chemicals. This review places more emphasis to nanomedicines. However, interested readers may refer to a previous article providing more information on aspects of occupational health and environmental safety [2].

In Europe, all chemicals including the excipients used in the production of pharmaceutical products fall under the authority of the European Chemicals Agency (ECHA) and underlie regulation (EC) No. 1907/2006 concerning Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) [24] as well as regulation (EC) No. 1272/2008 on classification, labelling and packaging of substances and mixtures [25]. These apply to all substances manufactured or imported at more than one ton per year [26] and requires the submission of a dossier describing the physicochemical, toxicological, and ecotoxicological characteristics [26]. The second article of REACH explicitly excludes drug substances, medicinal products, and invasive medical devices which fall under the authority of the EMA [24]. They are thoroughly evaluated during the process of drug approval on a case-by-case basis.

However, the pharmaceutical production chain is also affected by limited availability of materials and by restrictions in the exchange of interim products between different production sites. All bulk materials of a

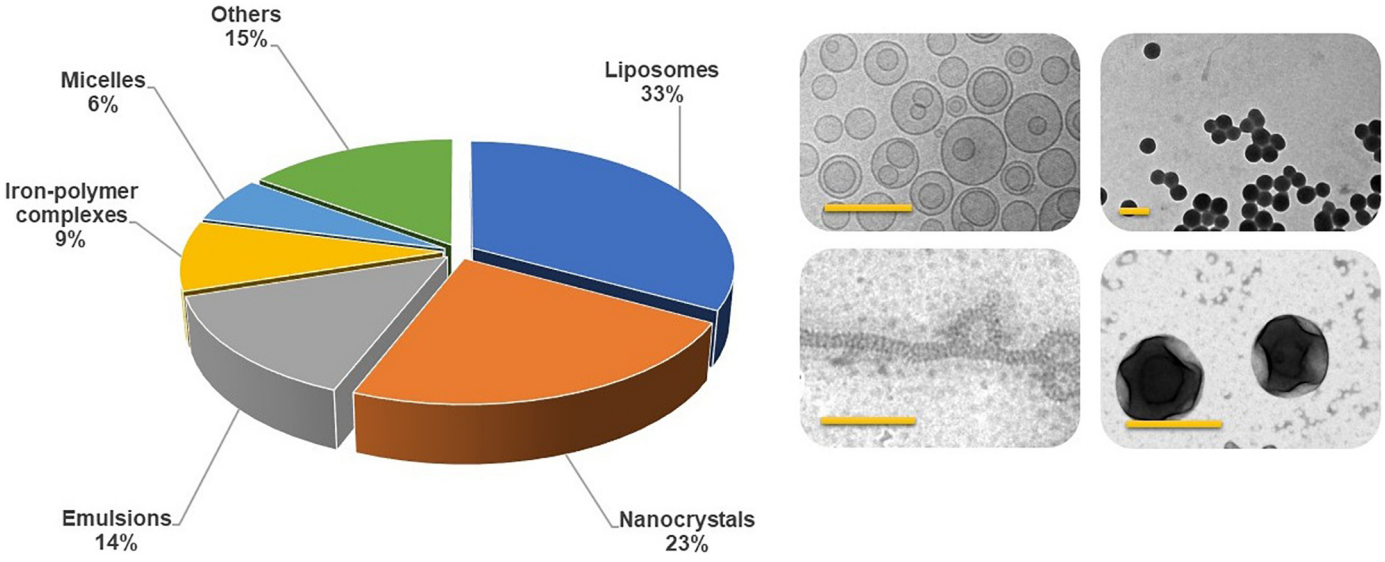

Fig. 1. Pie chart of nanotechnology-related platform technologies identified in CDER submissions (left side, figure modified from [3]) and cryogenic electron micrograph of liposomes (right side, left upper corner) and polymeric micelles (right side, left lower corner) as well as transmission electron micrographs of polymer nanoparticles (right side, right upper corner) and polymer-stabilized nanocrystals (right side, right lower corner). In all micrographs the yellow bar indicates a distance of 200 nm.

Table 2
Common terminology and definitions used by regulators and the scientific community.

Nanomedicine	Nanomedicine often refers to nanotechnology-based applications for prevention and treatment of diseases. According to the National Institute of Health (USA), nanomedicine is an offshoot of nanotechnology, which refers to highly specific medical intervention at the molecular scale for curing disease or repairing damaged tissues, such as bone, muscle, or nerve.
Nanocarrier	Nanocarrier often refers to drug delivery systems in the nanoscale which alter the biodistribution or penetration behavior of a compound by taking advantage of the physicochemical features of the carrier including the particle size or the surface structure.
Non-biological complex drug	A non-biological complex drug refers to a medicinal product, not being a biological medicine, where the active substance is not a homo-molecular structure, but consists of different (closely related and often nanoparticulate) structures that can't be isolated and fully quantitated, characterized, and/or described by physicochemical analytical means. It is often used in Europe referring to nanocarrier formulations such as injectable liposomes or nanoparticles.
Engineered nanomaterials/Intentionally manufactured nanomaterials	Engineered nanomaterials often refers to any material in the nanoscale that has been intentionally manufactured. The term excludes all naturally occurring materials and has been used by several authorities including the US-FDA and the European Food Safety Authority.
Drug product/Medicinal product	According to the US-FDA, drug products are "finished dosage forms, for example, tablets, capsules or solutions that contain an active pharmaceutical ingredient, generally, but not necessarily, in association with inactive ingredients". According to EMA, medicinal product is "a substance or combination of substances that is intended to treat, prevent or diagnose a disease, or to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action".
Nanocrystal	Nanocrystal often refers to a drug crystals in the nanoscale which are often produced by milling or homogenization processes.

certain volume that are either imported or delivered within the European Economic Area need to be registered as chemicals [24]. This includes intermediate processed raw materials sold to pharmaceutical companies and puts more pressure on the subcontracted manufacturers.

A number of guidance documents specific for nanomaterials were issued by the ECHA in 2012 [6–8] and 2017 [10–12]. They explain the requirements for registration and testing of chemicals including nanomaterials within the definition recommended by the European Commission (EC) [4]. On 3rd of December 2018, the European Commission adopted new requirements for the registration of nanomaterials. According to the new requirements, both company and ECHA will be required to perform a risk assessment of nanomaterials. These amendments to REACH will come into force on 1st of January 2020 [27].

Since the European nanomaterial definition (see Table 1) does not exclude nanomaterials incidentally formed during the manufacturing process, there are concerns that pharmaceutical manufacturers will not be able to measure the purity of their bulk materials with regards to the presence of nanomaterials and, consequently, will have to go through such a risk assessment. Consequently, these new requirements can potentially form a trade barrier for the import of novel excipients and weaken the European economy.

In the US, a wide range of nanomaterials fall under the Toxic Substances Control Act (TSCA) [13] which was recently amended by the Frank R. Lautenberg Chemicals Safety for the 21st Century Act [14] (see Fig. 3). They are subject to specific requirements including an information gathering rule on new and existing nanomaterials and premanufacture notifications for nanomaterials. Following the TSCA section 8 (A), manufacturers must report the specific chemical identity, production volumes, methods of manufacture, processing, use, exposure and release information as well as available health and safety data [28]. Many pharma-grade excipients fall under this new legislation and must comply with these rules. A draft guidance has been issued by the US-EPA in January 2017 describing frequently asked questions and answers the agency has received from manufacturers of nanoscaled materials [29].

The Asian chemicals market is widely dominated by China, the world's second largest economy. In 2004, the Standardization Administration of China (SAC) issued a definition of nanomaterials (see Table 1) [2,16]. Similar efforts have been made in Japan and South Korea [2].

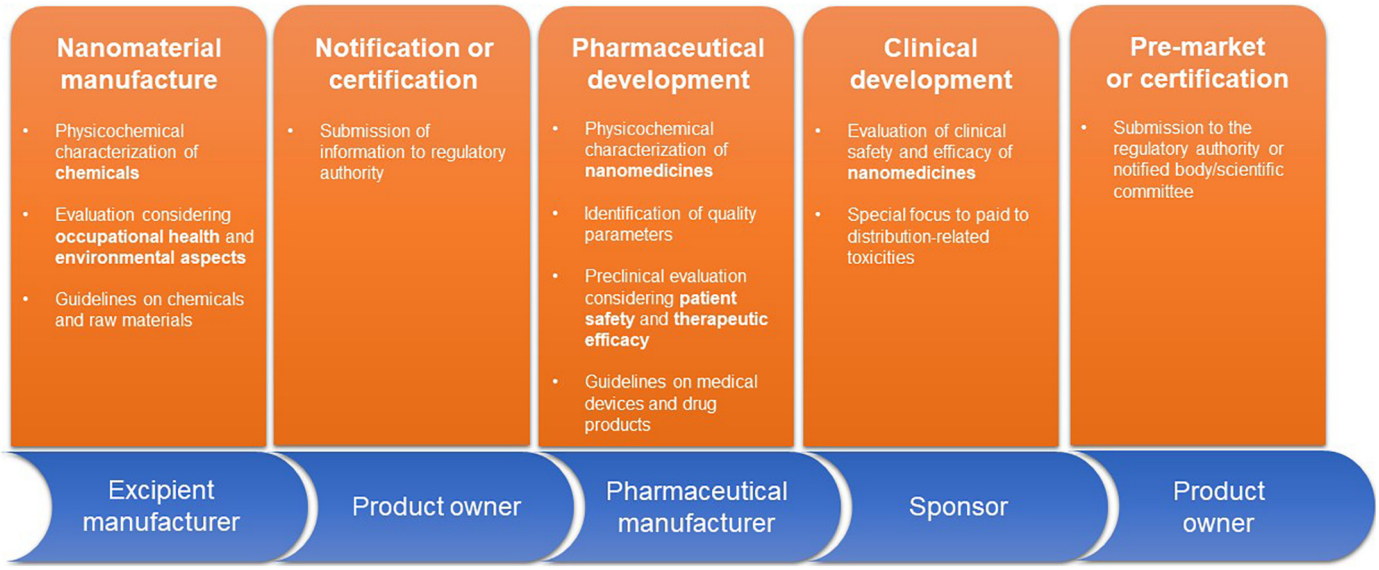

Fig. 2. Flowchart of the processes involved in the marketing and commercialization of nanomedicines with emphasis to the procedures in USA and EU.

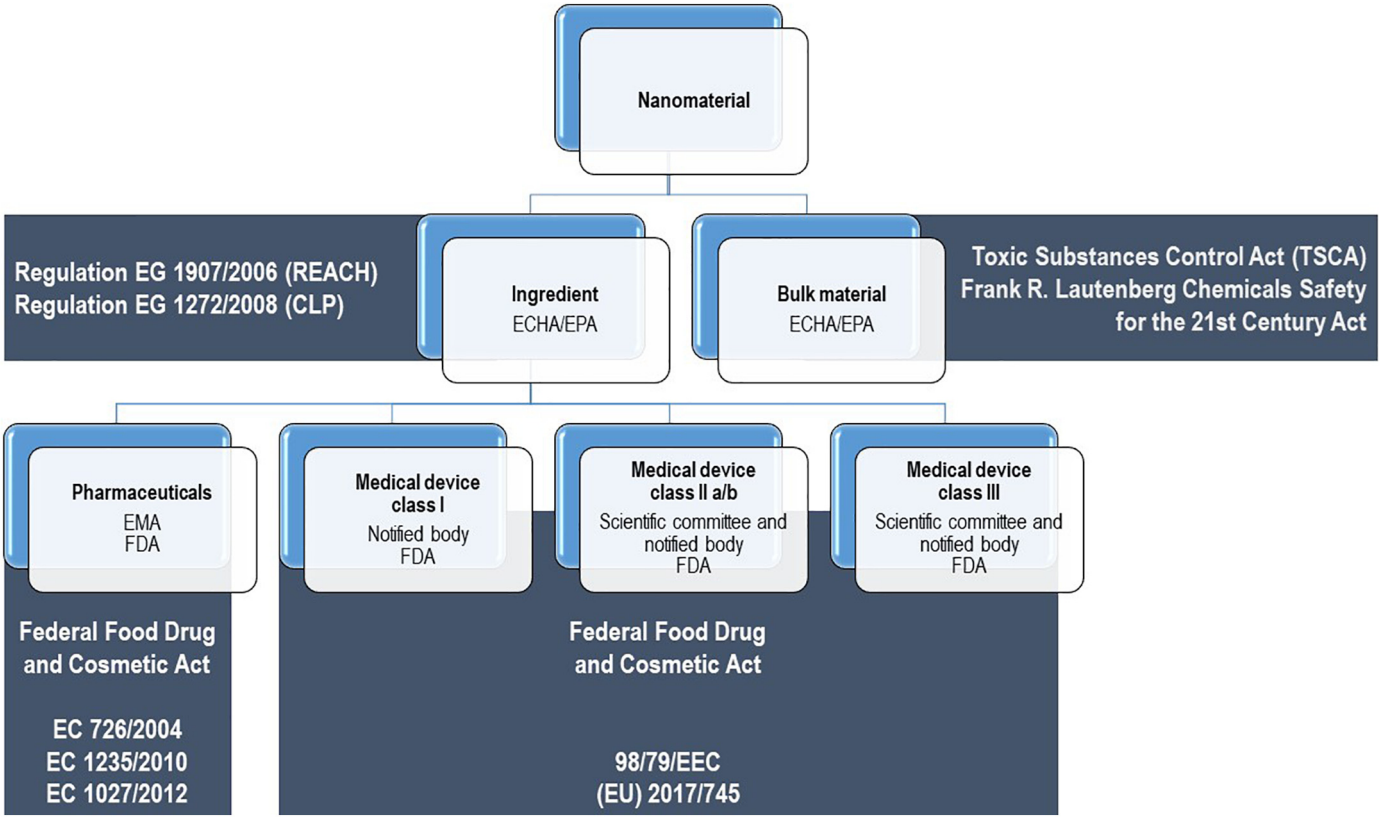

Fig. 3. Regulatory framework in Europe and the USA applied to the pharmaceutical product chain including ingredients, pharmaceuticals and medical devices.

For China, premarket-registration at the Chemical Registration Centre of the Ministry of Environmental Protection is required [30]. So far, there are no major changes to the status quo as presented in a previous article in 2016 [2], whereby, the Chinese government places more focus on the economic growth of nanotechnology [31]. However, there is an ongoing development of standards and protocols for nanomaterial characterization to strengthen the national nanotechnology industry [2]. Other Asian countries are still in the evaluation process and did not present much progress in the regulatory landscape.

In India, the Ministry of Science and Technology has released draft guidelines and best practices for safe handling of nanomaterials in research laboratories and industries in February 2016 [32]. Although these guidelines identify potential hazards from nanomaterials they do not provide any definitions for nanomaterials and state that the Nano Mission is currently in the process of drafting a set of regulatory guidelines for nanomaterials using the European regulatory framework provided by REACH as a model.

With regards to South American market, an initiative by the Brazilian Industrial Development Agency from 2013 [2] has not been followed by any further actions and the progress has been overshadowed by the ongoing economic crisis. Therefore, there are no further activities to regulate the nanomaterial market compared to the situation from 2016 [2].

From a global perspective, the decision for a high-technology manufacturing site (as it will be used for the manufacture of nanomedicines) is driven by the supporting infrastructures, the ability to recruit sufficiently qualified manpower, as well as by the availability of raw materials. Against this background, differences between the regulatory frameworks will affect the balance between the different markets and weaken the supply chains. Further, excipient manufacturers will sometimes provide smaller volumes of excipients to fulfill the requirements set by each regulatory framework which, in consequence, potentially increases production costs and, in some cases, may even outweigh the market value of some high-quality excipients.

2.2. Medical devices

Between different countries, there are only minor differences in the definitions applied to medical devices (see Table 3). However, in Europe, the USA, and China, the implementation of safety standards follows different procedures which are responsible for the huge differences between the national markets.

Recently, the European directives on medical devices [33] and active implantable medical devices [34] from the early 1990s have been repealed and the new regulations 2017/745 [35] and 2017/746 [36] were issued. After a short transition period, they will come into force in 2020 [35] and 2022 [36], respectively.

Rule 19 of the new directive [33] refers to the European definition of nanomaterials [4] and classifies all devices incorporating or consisting of nanomaterials as class II b or class III medical devices. Currently, market approval of medical devices is subject to accredited notified bodies but many of them lack the expertise to evaluate the clinical evidence presented by the manufacturer.

Against this background, the scrutiny procedure of the new directives [35,36] involves expert panels under the European Joint Research Center to assess the safety of implantable falling under class III and class IIb medical devices if they are used for the administration or removal of drug products. Combination or borderline products remain under the authority of the EMA.

A guideline from 2015 provides detail information on the requirements [37] (see Section 3). The aim of this directive was to implement a reliable regulatory structure to the medical devices market, but it has also caused great insecurity and ambiguity among the manufacturers on dossier submission. This was addressed by an update of the ISO 10993 standard, but there are still concerns that nanotechnology-related products will face a more restrictions affecting market entry and, consequently, product cost.

Table 3
Medical device definitions in the USA [38], China [39] and the EU [35].

		
"An instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including a component part, or accessory which is: 1. recognized in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them, 2. intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or 3. intended to affect the structure or any function of the body of man or other animals, and which does not achieve any of its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes"	*"Any instrument, apparatus, appliance, material, or other article whether used alone or in combination, including the software necessary for its proper application. It does not achieve its principal action in or on the human body by means of pharmacology, immunology or metabolism, but which may be assisted in its function by such means; the use of which is to achieve the following intended objectives: 1. Diagnosis, prevention, monitoring, treatment or alleviation of disease; 2. Diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap conditions; 3. Investigation, replacement or modification for anatomy or a physiological process; 4. Control of conception"*	*""Medical device' means any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of: diagnosis, prevention, monitoring, treatment or alleviation of disease, diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap, investigation, replacement or modification of the anatomy or of a physiological process, control of conception and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means;"*

The new framework is expected to harmonize the criteria for evaluation and acceptance of medical devices among the member states and to establish a transparent procedure for risk assessment. However, one major problem arises from a shift in the competencies from the notified bodies to the expert committees which will potentially reveal the knowledge gaps in the characterization of medical devices. Even though many of these devices have a long history in the market, a systematic approach to assess their safety based on objective scientific criteria (*e.g.* by conducting animal studies or clinical trials) can easily outweigh the market value. Further, the limited capacity of regulatory agencies and notified bodies to evaluate and approve such a high number of products may lead to delays in the registration process and, consequently, a loss of market share for some manufacturers.

The US market offers two alternative regulatory pathways for the registration of medical devices. As an alternative to the time-consuming pre-market approval, the Medical Device Regulation Act [38] allows manufacturers to provide evidence that their product is 'substantially equivalent' to another approved device [40]. The 510 (k) is a technical dossier which contains technical, safety and performance information. It turned out to be very flexible and does not follow a strict format hence it was more popular than the traditional premarket approval process [41]. This *'comparability approach'* is a cornerstone of the US medtech market and marks a major difference to the European approval system which puts more emphasis on the pre-market evaluation and testing.

A status report of the US-FDA from 2019 identified 2586 'nano implantable devices' among the medical devices sold in the USA [42] but only 36 of them had gone through the lengthier premarket approval process [42]. The US-FDA further supports the industry through the implementation of the online product classification database to help applicants select the right pathway for their products [43]. However, for high-risk devices intended to support or sustain human life, or prevent impairment of human health, or present a potential, unreasonable risk of illness or injury, they must be subject to the pre-market approval procedure.

In China, according to the People's Republic of China State Council Decree No. 650 [39], medical devices are subject to premarket approval by the Chinese State Food and Drug Administration (CS-FDA). Recently, the Chinese Ministry of Justice published draft amendments to the current medical device directive to reduce hurdles for market entry. All products containing nanomaterial products are classified as class III medical devices and have to undergo clinical evaluation. However, the

proposed changes to the current regulatory framework adopt a simplified approach to the requirements to prove clinical equivalence by allowing the use of clinical data from foreign countries to gain marketing authorization in China [44]. So far, there are only two guobiao (GB) standards in place which are specific for medical devices [45,46].

Only a few other Asian countries appear to be active in the development of a jurisdictional framework to specifically address nanomaterials. In the 2000s, Japanese ministries have been promoting research in safety measures and risk assessment urging the industry to conduct voluntary safety surveys. However, medical devices are still regulated under the general regulatory framework without any more specific rules for nanomaterials. The same applies to South Korea where there is only the issuance of a nanomaterial definition.

In India, medical devices are governed by Central Drugs Standard Control Organization (CDSCO) and at the time of writing this review no regulations or guidelines have been provided by CDSCO or Government of India.

The multilateral trading systems which supported decades of economic growth make the differences in the regulatory landscape more apparent. At present, to comply with the European standards and safety regulations, manufacturers must go through a time-consuming certification process which raised concerns in the pharmaceutical industry. The US system allows more flexibility and, once 510(k) clearance is attained, manufacturers gain access to the market but they are also subject to facility inspection which enforces compliance with US-FDA standards hence carry high litigation risks.

Recently, foreign companies which have been facing significant barriers to the Chinese medical devices market due to strict regulations regarding foreign investment into the national industries, are now seeing the first indications of a paradigm shift. This is in line with the current strategy of the People's Republic of China to advance novel technologies and transform into a center of research and innovation.

2.3. Pharmaceutical products

To cover the European market, the EMA uses a working definition for nanomedicines which differs from the EC's existing recommendations on definition of nanomaterials [4]. All products and devices purposely designed for clinical applications with at least one component in the nanoscale and which exhibit specific properties and characteristics related to nanotechnology and associated to expected clinical advantages fall into that range. In addition, the EMA has issued a number of reflection papers on the requirements for nanomedicinal products for human use [47], generic liposomal products [48], surface coatings of nanoscaled parenteral dosage forms [49] and diagnostic iron oxide nanoparticles [50] have been published. Similar efforts have been made by the Japanese Ministry of Health, Labour and Welfare (MHLW) together with EMA in 2013, by providing a joint guidance on micelle formulations [51]. Additionally, in 2016, the MHLW issued its own guideline on the development of liposomes [52].

The first actions taken by the US-FDA related to the uprising nanotechnologies market dated back to 1997 when the first draft guidance on liposome drug products was issued. Recently, in April 2018, a final version of the document was published [53]. It defines liposomes as "*vesicles composed of a bi-layer (uni-lamellar) and/or a concentric series of multiple bilayers (multi-lamellar) separated by aqueous compartments formed by amphipathic molecules such as phospholipids that enclose a central aqueous compartment.*" [53].

Although liposomal vesicles are unique among the nanocarrier platforms presented in Fig. 1, many of the analytical methods and quality measures applied to liposomes translate to other nanocarriers (see also Section 3.3). Nonetheless, with respect to the growing diversity of the market, two more documents containing guidance for industry were released by the US-FDA, explaining the identification [15] and characterization [54] of nanotechnology-related products.

In South America, the Brazilian Health Surveillance Agency (ANVISA) formed a committee which comprised of experts from the nanomaterials niche area with focus on drugs, medical devices, food, hygiene products as well as diagnostic equipment and supplies. The aim was to develop a questionnaire for manufacturers willing to register products that contain nanomaterials. However, the initiative ended in 2016 without further actions. Up till now, all medicinal products containing engineered nanomaterials are evaluated on a case-by-case basis. Recently, in January 2018, the Nanotechnology Working Group of the Argentinian National Administration for Food, Drugs and Medical Technology (ANMAT) released a document summarizing the assays for characterization and comparability of nanomedicines [55]. The document was open for consultation until February 2018 and seminars and trainings between the ANMAT and the pharmaceutical industry had completed. At the time of writing this article, the final version of the document is still being finalized. Most other South American countries refer to the international guidelines and do not provide more detail information.

In Asia, a wide variety of GB standards support the industry in reporting the quality features of nanomedicines [56] with participation from major regions such as China and Japan. The Japanese MHLW in collaboration with EMA has published a reflection paper on polymeric micelles [51] as well as its own guideline for the development of liposomes [52].

In March 2019, the Indian Ministry of Science and Technology [57] issued a first draft guideline for the evaluation of nanopharmaceuticals [57]. It applies to engineered nanomaterials and excludes medical devices, *in vitro* diagnostics, tissue engineered products as well as cell based therapy products. According to the guideline, 'nanopharmaceuticals' are defined as products containing nanomaterials in a range between 1 and 100 nm. A product is also deemed nanopharmaceutical if it falls in the range of 100 nm to 1000 nm and has altered pharmaceutical characteristics owing to the use specific size-related characteristics. As it is the case with traditional drug products, marketing authorization of all nanopharmaceuticals requires a case-by-case evaluation.

3. Requirements under the regulatory framework

Encouraged by a competitive market, the pharmaceutical industry has traditionally placed a strong focus on the process of drug design with emphasis on the economic outcome [58,59]. As a consequence, regulatory authorities define the product requirements as part of a growing regulatory framework and issue guidance documents based on current scientific standards [58]. In the following, the requirements applied to nanomedicines and nanomaterials will be discussed in more detail.

3.1. Excipients and drug substances

The US chemicals industry has only few restrictions for nanotechnology-related excipients but carries significant litigation risks which are responsible for its self-regulatory environment [2,60]. Commonly, the manufacture of new excipients requires a premanufacture notice and, for those excipients which are being used in drug products, a draft guidance has been issued by the US-FDA in 2014 [54].

In this context, the authority proposes a risk-based approach to define the quality levels required for nanotechnology-related products [54]. Conventional use of nanoscale excipients or other materials as well as all nanomaterials incidentally formed during the manufacturing process are excluded from the definition [15].

The guidance covers a wide spectrum of products including those for topical, oral, intravenous, subcutaneous and inhalation route of administration [54] but hardly supports the complex process of drug development. The key characteristics required to sufficiently describe these nanomaterials in pharmaceutical products are summarized in Table 4.

Table 4
Summary of the requirements applied to nanomaterials and liposomes according to respective regulatory framework in USA and EU.

United States of America	Europe
Nanomaterials	Nanomaterials [61] • Chemical composition (not specific for nanomaterials) • Particle size or volume specific surface area (VSSA) • Shape and morphology • Surface chemistry
Nanomaterials in drug products [54] Minimum requirements: • Chemical composition • Average particle size and size distribution • Shape and morphology • Chemical and physical stability Additional features: • Assay and distribution of any active ingredient associated with the nanomaterial and free in solution, • Structural features that relate to function • Surface properties • Hydrophobicity • Roughness • Coating properties, porosity • Particle concentration in vitro release • Crystal form • Impurities • Sterility and endotoxin levels	
Liposomal drug products [53] • Chemical composition including complete documentation of manufacture for all lipid components and other critical excipients naming sources and chemical quality, stability and purity • Morphology of the liposomes including lamellarity • Surface characteristics, e.g., pegylation • Net charge (e.g. zeta potential) • Drug product viscosity • Drug encapsulation efficiency • Drug loading • Particle size (mean and distribution profile) • Phase transition temperature • In vitro release of the drug substance from the liposome drug product • Leakage rate throughout shelf life • Liposome integrity changes in drug release, drug encapsulation • efficiency, liposome drug loading, size, e.g. in response to salt concentration, pH, temperature • Structure supported by spectroscopic or other analytical	(Generic) liposomal products [48] • Chemical composition, quality, stability features and impurities of carrier material and other critical excipients (e.g. lipids) • Morphology, mean size and size distribution and aggregates • Encapsulated fraction • In vitro drug release rate in physiologically relevant media • Reliable and discriminating validated in vitro release methods • In vitro leakage test in relevant media under multiple conditions • Stability on storage (and under proposed in-use conditions) • Robustness of reconstitution process

Giving a focus to the European situation, the REACH regulation affects the nanomedicines market by defining requirements for the characterization of chemicals which fall into the European nanomaterial definition (see Table 1). Submissions to the International Uniform ChemicaL Information Database (IUCLID) require a characterization of particle size, morphology and surface chemistry of the raw materials (see Table 5).

The registration using IUCLID is mandatory for all substances produced at a volume of >1 ton per year [61]. At present, previous dossier submissions are being retrospectively analyzed and new requirements are being defined.

3.2. Medical devices

For medical devices, the US-FDA offers premarket approval as an alternative to the 510(k) application [41] but only 1.4% of the nanotechnology-related products registered between 1980 and 2017 underwent the premarket approval procedure [42]. As a consequence, there is an strong influx of medical devices into the US market which were assessed using the 510(k) scheme [42]. Only for class III devices, which are deemed to have a strong impact on human health, is the pre-market approval pathway mandatory (see also Section 2.2).

On the contrary, the European regulations 2017/745 [35] and 2017/746 [36] define a mandatory pathway for premarket approval of medical devices based on objective scientific criteria. The methodology for an exposure-based risk assessment has been listed in a guidance issued by the Scientific Committee on Newly Identifed Health Risks (SCENIHR) in 2015 [37] and a broad array of methods are available to appropriately characterize nanomaterials (see Table 5). According to the European directive, medical devices are classified as class III if they present a high or medium potential for internal exposure, class IIb if they present a low potential for internal exposure and class IIa if they present a negligible potential for internal exposure [35]. The exposure is estimated based on the type of the medical device (*e.g.* surface-contacting device, external communicating device, implant device) and the contact time within three categories (≤24 h, 24 h to 30 d, >30 d). For devices with a medium to high risk of exposure, the an evaluation of toxicokinetics is required [37].

With regards to the Asian markets, until 2012, the CS-FDA approved 190 products within two categories of nanotechnology-related medical devices including nano-hydroxyapatite as a bone repair material, and silver-based anti-bacterial product for the treatmenent of wounds [62]. A total of 118 GB standards [63] are the cornerstone of Chinese nanomaterial regulations and provide more information on the mandatory and recommended characterzation methods. So far, only two standards on medical devices are issued by the CS-FDA and dealt with cytotoxicity testing [45] and endotoxin content [46]. All other standards provide information on the methodology without referring to a certain group of products.

Similarly, no further requirements have been defined by the authorities of South Amercian countries. So far, the EU has placed the strongest emphasis on the risk assessment as part of the regulatory process which can be seen as strong barriers for market entry.

Table 5
Requirements and methodology proposed for the characterization of nanomaterials in medical devices according to the European guideline [37].

Parameter	Methods
Chemical composition/identity	Mass spectrometry Atomic absorption spectrometry Fourier-transform infrared spectrometry Nuclear magnetic resonance Absorption spectrometry X-Ray diffraction Mass spectrometry Inductively coupled plasma mass spectrometry Gas chromatography/Liquid chromatography mass spectrometry Raman spectroscopy
Particle size	Field flow fractionation Hydrodynamic chromatography High performance liquid chromatography Analytical ultracentrifugation CLS disc centrifugation Dynamic light scattering Nanoparticle tracking analysis Electron microscopy Differential Mobility Particle Sizing
Physical form and morphology	Atomic force microscopy Transmission electron microscopy High resolution transmission electron microscopy Scanning electron microscopy Scanning transmission electron microscope Scanning tunneling microscopy Nuclear magnetic resonance X-ray diffraction
Particle and mass concentration	High performance liquid chromatography Absorption spectrometry Inductively coupled plasma mass spectrometry Gas chromatography/Liquid chromatography mass spectrometry Atomic absorption spectroscopy
Specific surface area	Brunauer-Emmett-Teller method
Surface chemistry	Mass spectrometry X-ray photoelectron spectroscopy Fourier transform infrared spectroscopy Nuclear magnetic resonance Analytical ultracentrifgation Suspended particulate matter Surface enhanded Raman spectroscopy Nanoscale secondary ion mass spectrometry Laser doppler electrophoresis
Surface charge	Phase Analysis Light Scattering
Redox potential	Potentiometric methods X-ray diffraction absorption spectroscopy
Solubility and partition properties	Solubility and dissolution rate in water and other solvents
pH	pH meter
Viscosity	Capillary viscometer Flow cup viscometer Rotational viscometer Rolling ball viscometer Drawing ball viscometer
(Aparent) density	Weight per volume of a dry powder
Dustiness	Continuous drop method
Chemical reactivity/catalytic activity	Kinetic measurements of chemical, biochemical and/or catalyzed reactions
Photocatalytic activity	Transmission electron microscopy UV absorption X-ray topography

3.3. Pharmaceutical drug products

The current regulatory frameworks provide authorities flexibility to set appropriate scientific standards to evaluate and approve nanomedical products on a case-by-case basis. The US-FDA, EMA [48], Japanese MHLW [52] and the Argentinian ANMAT [55] have published their current thinking on the documentation and characterization of nanomaterials outlining the product-specific quality and safety measures.

A comprehensive collection of recommendations is provided by the US-FDA guidance on liposomes [53] which reflects >20 years of experience with such products. It has been widely adopted by many regulators [48,52]. The requirements for physicochemical characterization are summarized Table 5.

The Japanese guidance also refers to liposomes carrying targeting moieties [52]. In cases where ligands are bound to the lipsome surface, the (conformational) structure, modification efficiency, and binding capability of the modified liposomes to the target cells should be investigated [52]. For the release assay, a biorelevant setup using blood or serum is explicitly highlighted [52].

4. Best practice guide for characterization of nanomedicines

The characterization of nanotechnology-related products requires a combination of different techniques to understand the physicochemical features and how these affect efficacy and product safety. While from a regulatory perspective, the raw materials (excipients) require assay systems to assess the environmental toxicity and effects on occupational health, drug products and medical devices follow their own framework putting more emphasis on therapeutic applications (see Section 4). A profound review of the current practices in cellular and molecular toxicology of nanoparticles is already presented in the current literature [120] as well as a summary of the current *in vivo* methods [121]. In the following, a brief summary of the current practice with focus on drug products and medical devices will be presented. Complementing the summary of current literature, the authors' recommendations for all areas of characterization can be found in Table 7.

4.1. Chemical characterization and stability

One key requirement in the production of drug products including those comprising nanomaterials, is the characterization of chemical structure, molecular weight and purity of the raw materials. This applies not only to the active pharmaceutical ingredient (API) but also excipients with a strong impact on therapeutic efficacy and safety [53,54] such as synthetic or semisynthetic polymers [51], lipid materials [48,53] or complexing agents [50]. As a consequence the block copolymers used in the manufacture of nanoparticles require careful analysis of the chemical structure. For example, the properties of poly-lactic-*co*-glycolic acid (PLGA) depend on the ratio of the two monomers.

Recently, the copolymer polyethylene glycol–polylactic acid has been used in the synthesis of the Accurins™ [122]. Before assembling the nanocarrier system, the chain length was determined by nuclear magnetic resonance (NMR) [122] and polydispersity was measured by gel permeation chromatography (GPC) [122]. More chemical characterization methods included the determination of residual solvents using gas chromatography (GC) as well as the quantification of the API by high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) methods [123].

To comply with the existing guidelines [48,52,53,55], HPLC can be applied to determine the purity of naturally-sourced lipid mixtures used in liposomal drug delivery [124]. Commonly, the composition of fatty acids of the triglyceride mixture as well as the total percentage of each triglyceride is reported [125]. The thresholds for reporting impurities follow the guidelines 3QA [126] and 3QB [126] of the International

Table 6
Selected drug products and medical devices registered in the EU or the USA. For the authorization in Europe the centralized procedure through the EMA (former EMEA) or authorization by at least one member state was included. A superscript a indicates that the product is currently out of market.

Trade name	Description	EU Product type (authorisation)	US Product type/(authorization)	Ref.
Abelcet	Amphotericin B lipid complex for the treatment of fungal infections (intravenous administration)	Medicinal product (1995)	Drug product (1995)	[64–66]
Abraxane®	Paclitaxel nanocrystals comprising human serum alumin as a colloid stabilizer (intravenous administration)	Medicinal product (2008)	Drug product (2005)	[67,68]
Acticoat Flex 3 and 7	Wound dressing impregnated with silver nanocrystals (topical administration)	Medical device (2009)	Medical device (2009)	[69–71]
Ambisome®	Amphothericin B liposomal formulation for the treatment of fungal infections (intravenous administration)	Orphan medicinal product	Drug product (1997)	[72–74]
AVflo™ Vascular Access Graft	Nanofibrous polyurethane vascular access graft for hemodialysis patients (implantation)	Medical device (2008)	Medical device (2008)	[75]
Betalutin™	Murine anti- CD37 antibody conjugated to the beta-emitting isotope lutetium-177 (intravenous administration)	Orphan medicinal product	Investigational drug product	[76–78]
Bydureon®	Exenatide nanocrystal formulation for the treatment of type 2 diabetes (subcutaneous administration)	Medicinal product (2011)	Drug product (2012)	[79–81]
CELLSEARCH® Circulating Tumour Cell Kit	Immunomagnetic kit to detect circulating cancer cells from blood samples (*ex vivo*)	Medical device (2004)	Medical device (2004)	[82]
DaunoXome	Liposomal formulation of daunorubicin citrate (intravenous administration)	Medicinal product (1995)	Drug product (1996)	[83]
DepoCyt(e)®	Sustained-release liposomal formulation of cytarabine utilizing the DepoFoam® technology (intracerebroventricular administration)	Medicinal product (2001)	Drug product (1999)	[84]
DepoDUR®	Liposomal depot formulation of morphine for the treatment of pain (epidural administration)	Medicinal product (2006)	Drug product (US 2004)	[85,86]
Doxil® (US)	Doxorubicin liposomal formulation of modified with poly ethylene glycol	Medicinal product (2000)	Drug product (1995)	[87]
Caelix® (EU)	(intravenous administration)			
Eligard	*in situ* implant formulation of leuprolide acetate for the treatment of prostate cancer (subcutaneous administration)	Medicinal product (2007)	Drug product (2004)	[88,89]
Emend®	Aprepitant nanocrystals for the treatment of nausea (oral administration)	Medicinal product (2003)	Drug product (2003)	[90]
Feraheme™ (US)	Ironoxytol nanoparticules as an iron replacement (intravenous administration)	Medicinal product (2012)	Drug product(2009)	[91]
Rienso® (EU)				
Feridex™	Iron oxide nanoparticles for diagnostics (intravenous administration)	Medicinal product (1995[a])	Drug product (1996[a])	[92,93]
Fresenius Polysulfone® Helixone®	Helixone® is the advanced high-flux polysulfone membrane of the FX-class of dialysers coated with nanoparticles (dialysis equipment)	Medical device (NA)	Medical device (2012)	
Gastromark™ Lumirem®	Diagnostic iron oxide nanoparticles used as a contrast agent (oral administration)	–	Drug product (1996[a])	[94,95]
Invega® Sustenna™/Xeplion	Paliperidone palmitate nanocrystal formulation for the treatment of schizophrenia (intramuscular injection)	Medicinal product (2007)	Drug product (2009)	[96]
KADCYLA®	Antibody drug conjugate comprising trastuzumab	Medicinal product (2013)	Drug product (2013)	[97,98]
LiPlaCis®	Liposomal formulation of the anticancer agent cisplatin (intravenous administration)	Investigational medicinal product	Investigational drug product	[99,100]
Megace®ES	Megestrol acetate nanocrystals for the treatment of anorexia (oral administration)	–	Drug product (2005)	[101]
Mepact®	Liposomal formulation of the immune modulator mifamurtide used for cancer therapy (intravenous administration)	Medicinal product (2009)	–	[102]
MidaForm™-Insulin-Pharmfilm®	Gold nanoparticle based needle-free transbuccal delivery system for insulin	Investigational medicinal product	Investigational drug product	
Myocet®	Liposomal formulation of doxorubicin for cancer therapy (intravenous administration)	Medicinal product (2000)	Drug product (2000)	[103,104]
NanoTherm	Iron oxide nanoparticles for thermotherapy of tumors using a magnetic field (intratumor injection)	Medical device (2017)	Investigational device	[105]
NanoXray	Hafnium oxide nanocrystals designed to increase the dose and efficacy of radiotherapy inside a tumour without causing additional damage to surrounding healthy tissues	Investigational medical device	Investigational drug product	
NicAlert®	NicAlert® is intended for *in vitro* diagnostic professional use for the semi-quantitative determination of cotinine in urine.	Medical device (2006)	Medical device (2002)	[106]
Marqibo™	Liposomal nanocarrier formulationfor cancer treatment (intravenous injection)	–	Drug product (2012)	
Onivyde™	Liposomal formulation of irinotecan for cancer therapy (intravenous liposomes)	Medicinal product (2016)	Drug product (2015)	[107]
PegIntron	Interferone alpha-2b derivative modified with polyethylene glycol (subcutaneous administration)	Medicinal product (2000)	Drug product (US 2001)	[108]
Pexa-Vec	Modified Wyeth strain vaccinia poxvirus used as an oncolytic virus system (pexastimogene devacirepvec)	Investigational medicinal product	Investigational drug product	[109]
Risperdal® Consta®	Risperidone nanocrystals for the treatment of schizophrenia (intramuscular injection)	Medicinal product (2008)	Drug product (US 2003)	[110]
Sienna+®	Coated superparamagnetic iron oxide nanoparticles for cancer diagnosis	Medical device	Medical device	[111–113]

Table 6 (*continued*)

Trade name	Description	EU Product type (authorisation)	US Product type/(authorization)	Ref.
	(subcutaneous injection)	(2013)	(2015)	
TG6002	Oncolytic viral immunotherapy (intravenous injection)	Investigational medicinal product	Investigational drug product	[114]
TiLOOP® TiLENE®	Polypropylene implant with a titan nanoparticle coating (implantation)	Medical device (NA)	Medical device (NA)	[115]
Triglide™	Fenofibrate nanocrystals for the treatment of hyperlipidemia (oral administration)	–	Drug product (2005)	[116]
Verigene®	Gold nanoparticle kit for diagnostics of biomolecules *(ex vivo)*	Medical device (2011)	Medical device (2012)	[117]
Visudyne®	Liposomal formulation of verteporfin for photodynamic therapy (intravenous administration)	Medicinal product (2000)	Drug product (US 2000)	[118]
Vivitrol®	Nanocrystal formulation of naltrexone for the treatment of opiod addiction (subcutnaneous administration)	Medicinal product (NA)	Drug product (US 2006)	[119]

Council of Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH).

Noteworthy, most changes in the surface structure of nanocarriers occurs at an early stage by producing easy-to-characterize raw materials rather than modifying the surface after assembly of nanocarriers. In this context, the technologies available to characterize the raw materials are more reliable and comply with the current standards applied to drug products.

As an exception, poly(ethylene glycol) (PEG) crosslinkers have been widely applied in the surface functionalization of nanomedicines but the reactivity of PEG can vary from manufacturer to manufacturer and/or even from batch to batch. The quantitative reaction of maleimide with cysteine and determination of the unreacted thiol groups using Ellman's reagent provides a relatively simple solution for the quantification of maleimide-functionalized PEG molecules and the determination of PEG binding efficiencies [127].

4.2. Morphology and shape

The excipients used in medicinal products or medical devices may be rapidly degraded or excreted from the human body. However, the experiences with asbestos fibers in the 1970s served as a landmark reference for the evaluation of the toxicological features of chemicals which includes an analyses of morphology and shape. Asbestos fibers have been widely applied as an inert material in consumer products which an approximate exposure of 11 millions individuals in the US to between 1940 and 1978 [128]. The needle-shaped fibers are phagocytized in the lungs and puncture the cell membrane leading to a strong inflammatory response [129]. Against this background, the SCENIHR proposes the use of particle imaging technologies to characterize substances for properties, relevant to the toxicokinetic profile [37].

However, some particle characterization methods are affected by the morphological features of nanoparticles. For example, dynamic light scattering (DLS) and laser diffraction technique are based on spherical particles leading to intensity fluctuations of light scattered at the particle surface [130] which correlate to particle mobility and morphologies. Therefore, other than spherical shapes may change the light scattering patterns and lead to false results [130]. In addition, strong surface charges can affect the mobility due to a larger hydrodynamic diameter [131,132] such as in the case of superparamagnetic iron oxide nanoparticles [131,132].

Following the European recommendation for a definition of nanomaterials [4], an imaging technology is required to identify the external dimensions of the smallest unit of an agglomerate or aggregate (see Fig. 4, left). Electron microscopy has been widely applied to characterize the morphology of nanoparticles, liposomes and micelles [133–137]. The techniques include transmission electron microscopy (TEM) [133,136], scanning electron microscopy (SEM), atomic force microscopy (AFM) [136] and scanning transmission electron microscopy and scanning tunneling electron microscopy. It should be noted that as TEM and SEM involve staining and drying procedures they may lead to altered morphologies [137] (see Fig. 4, right).

Cryo-TEM is a much more advanced but expensive technique to characterize vesicles or other self-assembling structures near the native state [137]. Alternatively, the OECD recommends that AFM is suitable for characterization of solutions or dispersions [138]. However, this technique requires a high level of expertise [138].

There are no specific recommendations provided by the regulatory authorities describing on which technology to use for a certain nanotechnology-related product. For each product, the best available technique should be applied. In practice, only few methods qualify for routine quality control in terms of reproducibility and robustness. In most cases, morphology and shape are determined during early formulation or process development to verify the outcome by particle size measurements (*e.g.* DLS).

4.3. Polymorphic forms

First introduced by McCrone in 1965 [139], polymorphs occur for most compounds in the solid state. They contain molecules of a single chemical nature but exhibit different physicochemical properties including the melting point and drug solubility. The polymorphic form of an API affects most of the physicochemical properties and dissolution behavior [140]. Although it should be noted that even excipients can exhibit polymorphism affecting the physicochemical properties of the product *e.g.* Compritol 888 exhibits three different polymorphs affecting stability and drug release from nanoparticles [141].

Due to the widespread availability in academic and industrial settings, x-ray diffractometry and differential scanning calorimetry (DSC) are the most commonly applied techniques to analyze polymorphic forms [142]. This applies not only to most nanocrystal formulations [143,144] but also to liposomal carriers [145].

In recent years, more attention has been paid to lipid-based liquid crystals which exhibit some potential in drug delivery by providing a release matrix and protecting peptides, proteins, and nucleic acids from chemical and physical degradation [146]. DSC measurements can be used to determine phase transitions of phospholipids. Depending on temperature and the presence of other molecules, bilayers can exist in the gel phase, the rippled phase and the liquid crystalline phase each of which is characterized by a different release behavior [147].

In this context, the polymorphic form and phase transition behavior has a strong impact on the release properties of nanocrystals and liposomal formulations [148] and has been included by the US-FDA into their recent guidelines [53,54].

Table 7
The authors' recommendations based on our own experiences working in the area of nanotechnology-related products.

The authors' recommendations	
Chemical characterization and stability	A wide variety of nanomaterials is assembled using several chemical components and reagents which, together, form a physically stable nanostructure. One major challenge lies in the characterization of the end product which is largely defined by a combination of chemical and physical features. In this context, the threedimensional structure often plays a major role for clinical efficacy. Currently, most manufacturers synthesize well-defined and chemically pure raw materials (*e.g.* lipids, block copolymers, active pharmaceutical ingredients) and process these materials into a homogeneous monodisperse nanostructure (*e.g.* nanocrystal, liposome). More nanomaterials are generated by the emerging biopharmaceuticals industry which introduces distinct chemical modifications into large drug molecules to alter biodistribution or add more functionalities. Noteworthy, these two strategies arise from a lack of suitable combination methods which provide more information on the structural and quality features of nanomaterials. To develop such methods or to find ways of assembling the raw materials in a more predictable manner are two major challenges in the development of nanotechnology-based products. For the time being, we recommend to take advantage of one of these two strategies which already have been successful in translating nanomedicines from bench to bedside.
Morphology and shape	The investigation of morphology and shape is commonly accomplished during formulation development and will not be repeated in routine quality control. Depending on the structure and properties of the material (*e.g.* spherical micelles, fibers, softness) an optimal method should be identified. In most cases, cryo-TEM is regarded as the gold standard, however, more information on the surface properties can also be obtained by AFM. Noteworthy, the morphology and shape of the material affects the capability of the analytical setup to determine quality features such as the particle size. Consequently, the morphology should be determined again for post-approval changes and scale-up.
Polymorphic forms	Understanding the polymorphic forms of the excipient material and the drugs employed for formulation development may be the right strategy but the limitated sensitivity of DSC or XRD for smaller amounts of crystalline materials should be taken into account. A good strategy is to perform such measurements with several references including the physical mixture of all materials over a certain concentration range.
Particle size, size distribution and surface area	It is necessary to characterize nanotechnology-based products with techniques capable of identifying particles not only in submicron range but also micron and visible range. More often dilution of samples is required to perform such analysis and special attention should be paid to the colloidal stability of particles when preparing the dilution. For example, NTA often uses high dilutions. In such cases, a concentration range should be tested to obtain more information on particle stability. Another aspect that has to be taken into consideration is the morphology and background medium. As a consequence, '*morphology and shape*' as well as the '*in vitro stability*' have been included in most guidelines.
Surface properties	Electrical properties at the surface of nanoparticles are key indicators of the colloidal stability and surface modifications. The bulk zeta potential of a particle population can be measured by determination of electrophoretic mobility using DLS. Zeta potential of individual particles can be measured using Tunable Resistive Pulse Sensing. Noteworthy, the zeta potential easily changes in presence of ions or proteins. In many cases a titration of the material may be a good way to evaluate the behavior of the colloids in different environments (*e.g.* pH value, salt or protein concentration).
Drug load and encapsulation efficiency	Drug load and entrapment efficiency are critical quality attributes and therefore it is essential to identify how they are affected by different process parameters such as pH, buffer strength, temperature. Most effective methods for separating the bound fraction from the free drug include ultracentrifugation and size exclusion chromatography. When it comes to nanocarriers, maximizing the drug load is rather dangerous, as this strategy is likely to result in an large fraction of the drug loosely bound to the particle surface. Instead, a target drug load should be defined and encapsulation efficiency should be used through the optimization procedure.
In vitro stability and degradation	The *in vitro* stability is a rather important parameter as it provides a better estimate of the physicochemical features of nanotherapeutics *in vivo*. Nanotherapeutics are evaluated for multiple parameters after synthesis but, more often, this does not reflect reality in a physiological environment. How the physical properties of the formulation develop over time in physiological or biorelevant fluids is not only complementing the release experiments but also provides more the expected biopharmaceutical behavior. The methodology is difficult to develop and requires a deeper understanding of the physiological fluids employed during the measurement. A good advise to most researchers working with biological test systems (*e.g. in vitro* cell culture) is to include this parameter and to evaluate changes in particle size or charge in their cell culture media as well.
In vitro drug release	In quality control, the drug release method should be able to discriminate therapeutically relevant quality changes. Finding the most appropriate and cost-effective method often is an odyssey but often leads to a better understanding of the mechanisms of drug release. Our experiences indicate that the usual prescreening (*e.g.* solubility testing) accomplished when developing the assay provides some understanding but, in itself, uses its own methodology which may be different from the one used for the nanocarrier. Hence, these findings sometimes do not translate to the release test very well
Exposure scenario and risk assessment	The exposure scenario has been introduced in many guidelines but, taking a step back and having a look at the surrounding framework, there are several differences between the perspectives under which the exposure of workers, consumers or patients may come into play. There is not 'one exposure scenario' but multiple ways organisms may be exposed to nanomaterials. In Drug delivery, we often give an emphasis to biodegradability or excretability of the material for a certain administration route. In occupational health, inhalation of the material by workers at the manufacturing site are to be considered and, once the product has been applied and degraded, the environmental exposure can be a major concern. To follow a nanomaterial through the whole lifecycle of a product, the release of nanomaterials from the product but also the likelihood of disassembly should be taken into consideration. One way of doing that is to track the physical changes through the whole lifecycle. This challenge is still to be solved and requires more efforts within the scientific community.
Immunogenicity and pyrogenicity testing	While nanomaterials enable targeting of active ingredients to specific sites, they also become targets of the complement and mononuclear phagocyte systems as a result of their size. As there is currently limited knowledge to predict these interferences of the nanomedicinal products with the assay due to alterations to the physicochemical environment eg. pH, presence of chelating agents, serum, binding proteins and denaturants, appropriate controls should be introduced to the test setup.
Preclinical evaluation	Further understanding may be needed regarding the interactions of nanomaterials with biological systems. These interactions include, impact of intrinsic (*e.g.*, disease, age, sex) and extrinsic factors (*e.g.*, co-administered drugs) on exposure and response, the role of enzymes and transporters in their disposition, and their immunogenic potential.
Clinical evaluation	Nanomaterials can sometimes cross biological barriers in greater amounts than the larger particle size version. This can lead to increased safety concerns in some cases, such as increased penetration of the blood-brain barrier, or the placenta. If a drug product contains nanomaterials as excipients, including excipients that function as drug carriers, the biological fate of the carriers and their potential impact on safety may need to be determined in addition to those of the active ingredient. There may also be different risk considerations for different routes of administration *e.g.* phototoxicity with topically applied products; sensitization potential with subcutaneous administration; inhalational toxicity since smaller particles might be inhaled to greater extent; hemocompatibility with IV administration, accumulation with oral administration *etc.* Additional studies can be warranted if nanomaterial suggest the possibility of an altered effect in a particular tissue compared to previously approved drug product. In some cases, when the nanomaterial is not the active ingredient, assessment of its contribution to any observed toxicity could also be useful in interpret bridging studies. Therefore, inclusion of treatment groups with only the nanomaterial

Table 7 (*continued*)

The authors' recommendations	
	should be considered.
Biorelevant *in vitro* studies	When studying the *in vitro* performance under physiologically meaningful conditions, the driving forces for the drug to be released from the carrier have to be revealed. For many nanocrystal formulations, dissolution is most relevant, however, due to the small size of nanocarriers, it sometimes plays a minor role for nanocarrier delivery. In this context, the affinity of the drug to the carrier as well as a competition for binding sites when entering the physiological environment come into play.
In vitro-in vivo correlation	Most often, to establish *in vitro-in vivo* correlations for drug products, *in vitro* dissolution and human pharmacokinetic data is used. This current practice reflects our understanding of the mechanisms underlying peroral drug delivery and, in fact, most orally administered nanoformulations follow very similar rules. But they also present more challenges in the generation of meaningful dissolution data. Extrapolating this knowledge to other administration routes is dangerous and may be one reason for the poor success rate of nanomedicines. Neither the excessive use of sophisticated *in vitro* methods nor the more advanced analytical technology has led to a significant progress. Physiologically-based pharmacokinetic modeling is one of the most promising strategies to link the rather complex intertwined processes involved in drug delivery and to identify physicochemical parameters of interest from the existing preclinical and clinical data.
Pharmacokinetics and biodistribution	Current methodologies applied in preclinical and clinical research have been developed for drug molecules but sometimes hardly translate to the area of nanomedicines. Most pharmacokinetic studies from the early 1990s and 2000s quantify the total drug concentration in animal or human plasma. This sufficiently explains bioavailability of perorally or topically administered nanoformulations but hardly translates to nanocarrier formulations which occur in two fractions each of which is characterized by a different volume of distribution and clearance. To cover this complexity, the development of a method to separate different fractions present in the blood sample is advised. From each plasma sample, free and encapsulated drug can be quantified. If there is an infrastructure for conducting a more detailed analysis of the blood sample, which is often the case for preclinical studies, the association of the drug to certain fractions of blood cells may provide further information. Similarly, imaging technologies are widely available and can be used to identify distribution and elimination parameters more easily.

4.4. Particle size, size distribution and surface area

The particle size and size distribution are commonly reported to identify a nanomaterial and to characterize the end product of a pharmaceutical production process. Many techniques such as LD, DLS and nanoparticle tracking analysis (NTA) are based on the detection of scattered light. Compared to the LD technique which is often applied to measure particles in the micro range, DLS and NTA are particularly useful to analyze particles with a diameter below 100 nm (see Fig. 5). However, for materials in the lower nanometer range, NTA can detect only particles with a high refractive index.

As indicated by a review of CDER submissions submitted to the US-FDA, DLS is the most common technique for measuring the size of nanoformulations (see Fig. 5). However, a strong background signal as well as properties of the formulation which affect particle mobility in the dispersion medium (*e.g.* viscosity of the medium, high particle concentration) can change the results of the measurement. According to US-FDA, the validation of a DLS method involves testing for repeatability, precision, intermediate precision, and robustness [149]. Unfortunately, resolution of DLS to particle populations is rather limited and requires the populations to differ by factor of three [150]. NTA offers an alternative to the traditional DLS setup. The system is tracking particles by capturing the movement scattered light using a camera. However, the multiple settings and options as well as the lack of harmonized procedures require a trained and experienced operator [150]. Further, NTA requires particle concentrations in a range between 10^7 and 10^9 per milliliter while DLS has a broader range from approximately 10^8 to 10^{12} particles per milliliter [150]. Depending on the material and medium, each of these methods comes with a set of advantages and disadvantages. NTA combines particle mobility analysis with imaging technology and provides an very accurate number size distribution by tracking single particles in a polydisperse sample. However, the limited concentration range, a strong influence of the material properties (*e.g.* refractive index) and a poor reproducibility [150] make it less suitable for the daily routine. On the contrary, modern DLS systems can measure particles within a much broader concentration range and provide highly reproducible data which is affected by the material properties to a minor extent. [150]. However, the systems poorly respond to polydisperse samples.

Recognizing the technical challenge to quantitatively determine the size of a smaller particle fraction in polydisperse bulk materials, Kreyling and co-workers proposed a complementary definition using the volume specific surface area (VSSA) [151]. Dry powders with a VSSA larger than 60 m^2 per cm^3 can be classified as nanomaterials.

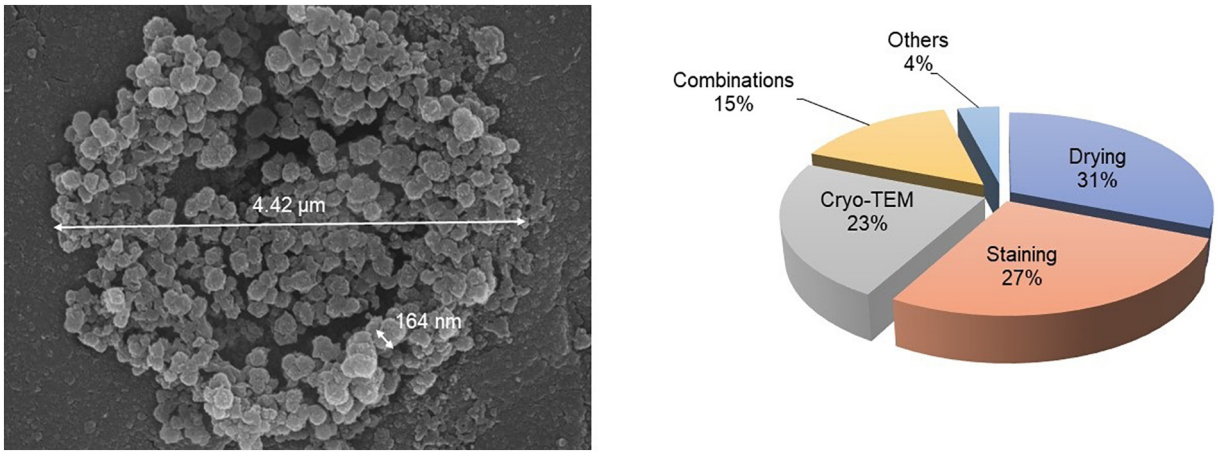

Fig. 4. Scanning electron micrograph of an agglomerate of Eudragit® nanoparticles prepared by glycofurol desolvation technique highlighting particle diameter and diameter of the smallest unit (left); evaluation of current literature using TEM for particle characterization (right) highlighting the techniques and sample preparation procedures (modified from [137]).

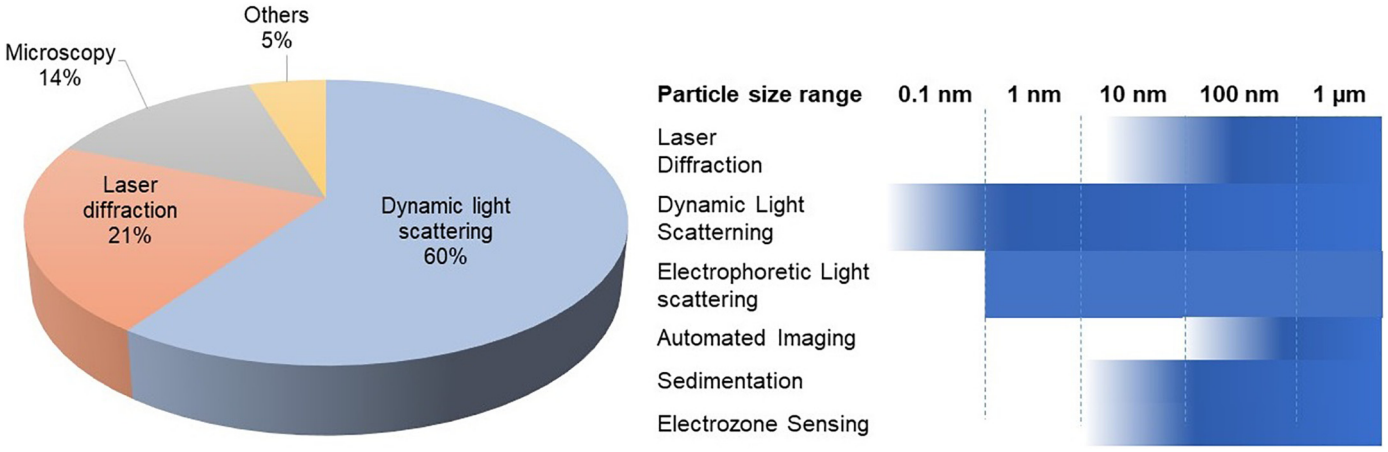

Fig. 5. Pie chart of the particle characterization methods described in CDER submissions to the US-FDA for drug products containing nanomaterials (modified from [22]) and particle size ranges of different analytical methods (modified from [156]).

The VSSA can be determined easily using the Brunauer-Emmett-Teller method [152–155] which is a well-known and cost-effective method and can be applied without further sample preparation. Further, it is tolerat to agglomeration and highly reliable [152].

4.5. Surface properties

A wide variety of aspects contribute to the physical stability of colloids including the surface structure, the presence of soluble stabilizers or surface coatings as well as the electrostatic repulsion.

Electrostatic repulsion occurs due to the presence of charged groups at the particle surface [157]. Conductometric titrations can be used to determine surface charge density [158], but they do not provide significant insight into particle aggregation and colloidal stability. Light scattering or laser Doppler velocimetry can be used to measure the electrophoretic mobility of particles in a medium and to determine the zeta potential [157]. Thomas M. Riddick introduced electrostatic repulsion as a criterion for colloidal stability, classifying particle systems by the zeta potential (see Table 8).

The zeta potential is often considered a key characteristic of nanomaterials with a strong impact on stability and cell interactions. To assure conductivity of the sample, the ISO and ASTM standards for zeta potential measurements suggest an aqueous 10 mM sodium chloride solution or comparable medium [159,160]. Based on the authors' experiences, the parameter reflects both, the properties of the delivery system as well as the medium used for the measurement. Against this background, its importance in drug delivery has often been overestimated and extrapolated from one microenvironment to another. However, the responses of the zeta potential to changes in ionic strength or pH value are affected by the surface chemistry and functional groups available at the particle surface. Within certain limitations they can be used as a fingerprint of the surface chemistry [161].

Further, in biomedical products the electrostatic repulsion is not the only aspect of colloidal stability. In some cases, the presence of steric stabilizers or coatings plays an even more important role. Zeta potential measurements may indicate the differences in surface-bound coating quantities, but will not detect unbound molecules. A chromatographic separation in combination with evaporative light scattering detector or charged aerosol detector can displace and separate the coating [124]. Another approach is the separation by asymmetric-flow flield-flow fractionation in line with multiangle light scattering or another suitable detection methods [54].

For some nanomedicines, the degree of surface coating or surface functionalization can be critical to the *in vivo* performance (see also Section 4.9.2). For most iron-carbohydrate complexes, the stability not only depends on surface charge but is also affected by the carbohydrate layer thickness. More advanced nanotherapeutics are using ligand structures to address specific tissues inside the human body. At present, there is no '*gold standard*' established to quantify the degree of surface functionalization of nanoparticles or liposomes. Depending on the nature of the nanomaterial, the optimal ligand architecture, stoichiometry and density per particle as well as the ligand distribution on the surface is assessed and optimized using *in vitro* biochemical and biological assays. The assays range from protein quantification assays to competitive enzyme-linked immunosorbent assay [162]. For inorganic nanomaterials, the ligand density may be determined by thermogravimetric analysis [163] or NMR [163,164]. However, in many cases, the determined ligand density does not correlate to the achieved *in vivo* response [54] although there are implications on biodistribution and the immunogenic profile of the formulation.

Table 8
Stability criteria for colloids according to Riddick [157].

Stability criteria	Average zeta potential [mV]
Maximum agglomeration and precipitation	±3
Strong agglomeration and precipitation	±5
Threshold for agglomeration	±15
Threshold for sensitive dispersions	±30
Moderate stability	±40
High stability	±60
Very high stability	±80
Extremely high stability	±100

4.6. Drug load and encapsulation efficiency

The drug load is the percentage of API in a drug formulation and defines the applicable dose range as well as the drug-excipient ratio which impacts the exposure to potentially toxic or immunogenic excipient materials. It differs from the encapsulation efficiency which is the percentage of the total API content encapsulated into nanomedicines. Current guidelines on liposomes [48,52,53] as well as the US-FDA's guidance on nanomaterials [54] include the drug load in the critical quality attributes.

The total drug content of the formulation is commonly determined using HPLC or UPLC methods [123] whereas quantification of the encapsulation efficiency requires a separation step. In the area of medical devices, there are some borderline products which contain drug substances. For example, the amount of nanocrystalline and dissolved silver plays a role with regard to release kinetics. In this context, a broad array of methods including syringe or centrifugal filtration [165,166], centrifugation [167] and solid phase extraction [168] have been reported. Leakage of API from the carrier can occur for liposomal

drug formulations and nanoparticles. Overall, the effectiveness of each separation technique depends on the properties of the specific nanomaterial. For instance, compared to nanocrystalline formulations, liposomes are more sensitive to shear stress and require careful separation, *e.g.* using a multi-step purification in centrifugal filters [167]. Even for some polymeric nanocarrier systems an extraction of API from the carrier has been observed when using syringe filtration [169].

The encapsulation efficiency is an important parameter and, in formulation design, provides an estimate of the drug-carrier affinity [170] and, consequently, stability of a formulation.

4.7. In vitro stability and degradation

The *in vitro* stability assay covers the physical changes of nanomedicines in response to an altered microenvironment rather than monitoring chemical changes as covered by the purity profile (see Section 4.1). The encapsulation procedure, storage, reconstitution, a change in the ion concentration [171] as well as the presence of physiological fluids and proteins can result in an agglomeration, erosion or degradation of the nanomaterial.

A wide variety of stability parameters can be used to describe the *in vitro* stability. Common methods include the measurement of particle size in presence of physiological fluids such as serum [170,172] or biorelevant media [173]. In some cases, the disassembly of colloids was monitored using more specific assays [174,175]. Goepferich and *co*-workers applied a Foerster resonance energy transfer assay to detect the stability of polymeric micelles in human serum [175]. The method was effective and robust to the presence of background signals and has been applied to a wide range of block copolymer formulations [174].

DLS and NTA are based on the intensity of scattered light and require a more careful interpretation of data [170,172]. Due to the presence of proteins, lipids and micelles, some of the physiological fluids applied to test the stability may not allow detection of size distributions by using these methods [172]. For accurate detection of particles from physiological fluids, intensity of the background signal should be tested and concentration of the analyte adjusted reasonably in order to achieve a good signal to noise ratio [174]. However, for some applications NTA may be suitable as it allows a background subtraction by applying a fluorescence filter for fluorescent particles.

4.8. In vitro drug release

Pharmaceutical dissolution testing plays a key role in the development and quality of peroral dosage forms. Biorelevant release assays reflect the physiological environment of the administration route by which the predictive power for the *in vivo* situation is optimized (see Section 4.10.1).

In quality control, release assays are applied to assess batch-to-batch variability as well as the variations in product performance following scale-up and post-approval changes. Economic aspects such as the volume and cost of the release medium and the duration of the assay have to taken into account [176]. The capability to discriminate between different batches and between a generic formulation and the innovator product are of major importance.

Many of the drug substances processed to nanotechnology-related products are poorly soluble. To achieve a total release of at least 80% of the drug, sink conditions are maintained by using higher volumes of dissolution medium [177] or the addition of surfactants [178]. Commonly used surfactants include emulsifying agents such as sodium dodecyl sulfate and polysorbate 80 [179,180] or cyclodextrins [181,182]. With regard to the technical setup, there is no 'gold standard' established for testing the drug release from nanosized particles [176]. A comprehensive review of the current literature and release technologies in formulation development and quality control has been published previously [176].

4.9. Risk assessment and toxicological profiling

The pharmacological and toxicological profiling requires a systematic evaluation of the effects of nanomaterials in a variety of *in vitro* and *in vivo* models. The assay systems widely depend on the functionalities of each component. For nanomaterials used in drug products, the degradation pathway and fate of each component under the conditions of drug therapy is evaluated on a case-by-case basis during the process of drug approval. The investigation of degradation and elimination pathways is based on the assumption of therapeutic use. However, with emphasis to medical devices, combination or borderline products, there is also a certain potential for the unwanted release of nanomaterials which does not follow the standards of drug therapy, *e.g.* by erosion of surface coatings and unwanted release of nanomaterials into systemic circulation. These cases are covered during risk assessment as part of the exposure scenarios.

4.9.1. Exposure scenarios and risk assessment

While an 510(k) application to the US-FDA often leads to a relatively quick market entry, the recent European guidelines on medical devices require a more detailed risk assessment, [35,183] and this applies to all medical devices comprising mobile or even immobilized nanomaterials [35]. For example, some wound dressings [69–71] or coated implants [115] potentially degrade during the time of use. The degradation can be monitored by applying standard methods of the International Organization of Standardization (ISO) for the biological evaluation of medical devices [184–189]. An assumption of nanomaterial exposure using current methodologies is difficult, but can be made based on expected time of tissue contact, *e.g.* skin, mucosal membrane, compromised surface, blood, and the duration of the contact within the three categories of limited contact (≤24 h), prolonged contact (>24 h to 30 days) or permanent contact (>30 days) [183].

Unfortunately, in presence of complex fluids, the quantitative determination of particulate matter cannot be achieved easily. Therefore, there are no assay systems in place to reliably detect release of nanoparticles. In this regard, the combination of a biorelevant setup with particle size characterization technology would be desired. Recent investigations applied NTA in dilutions of fetal calf serum to detect changes in particle size when exposed to physiological fluids [190]. Another study focused on the peroral route of administration and investigated nanomaterial agglomeration in biorelevant fluids [191].

4.9.2. Immunotoxicity and pyrogenicity testing

Though many drug products comprising nanomaterials are made entirely from substances that are generally recognized as safe (GRAS), some of them take advantage of novel, unique excipients that have not previously been tested in patients. Compared to the conventional formulation approaches, a rather complex combination of biotechnology-derived raw materials and small molecules may lead to unexpected health risks [54]. For this purpose, the immunotoxicological characterization is part of the preclinical safety evaluation. With regards to nanomedicines, this aspect is of particular importance for those products leading to a systemic exposure with the nanomaterial such as injectable diagnostics [192], drug products [103,104] or wound dressings [69–71].

In general, the immunotoxicity assessment of nanomedicines is based on existing framework for conventional drug products. The relevant ICH guideline focuses on the detection of direct immunotoxicity as a result of immunosuppression and immunostimulation [193]. An accepted study design in rodents is a 28 day study with consecutive daily dosing [193]. However, this method does not reflect the multitude of potential interactions between nanomedicines and the immune system.

Some of the adverse reactions have been related to the use of polymer coatings to prolong the half-life of nanocarriers including hydroxyethyl starch (HES) [194,195]. EMA and US-FDA advised against the use of HES in critically ill patients [196] due to increased mortality

and potential for kidney failure [56]. In comparison, PEGylation [197] has been more successful in a wide range of applications. Noteworthy to mention this high safety level has been reported for PEGylated non-targeted lipids which do not carry a ligand structure on the surface.

Another potentially life-threatening adverse reaction to nanomaterials is the complement activation-related pseudoallergy (CARPA) which is not readily detected when using the existing protocols. In some patients, CARPA occured after intravenous injection of the first dose of the liposomal formulation Doxil® [198,199]. Szebeni and co-workers proposed a porcine model to detect such adverse reactions but it is still under investigation [192].

Against this background, the safety of more advanced carrier materials should be carefully evaluated.

In routine quality control, the the contamination of samples with endotoxins is a known error-source in immunogenicity testing [200]. They can be detected usingt the Limulus Amebocyte Lysate assay [201–203], the Rabbit Pyrogen Test (RPT) [204] or the Monocyte Activation Test (MAT) [205,206]. Although an *in vitro* assay such as the MAT reduces the need of animal testing and may be more cost effective, it is not suitable for all nanotechnology-related products [207]. Further, some of the assay systems such as RPT and MAT are not endotoxin-specific. Other challenges involved with endotoxin testing might also occur due to potential interference of the nanomedicinal products with the assay due to alterations to the physicochemical environment eg. pH, presence of chelating agents, serum, binding proteins and denaturants [208]. As there is currently limited knowledge to predict these interferences, appropriate controls should be introduced to the test setup.

4.9.3. Preclinical evaluation

To assess the efficacy and safety of nanomedicines in a preclinical setting, the characteristics and quality of the formulation should be comparable to the final product [52]. This is even more important because, for many nanotechnology-related therapeutics, scale-up and freeze drying present major challenges [209]. Further, the pharmacological and toxicological profiling requires careful consideration of the desired administration route, dose range and indication of the drug substance. For example, wound dressings comprising nanosilver require an investigation of their potential in wound healing applications as well as a characterization of the expected penetration depth of the carrier into the wound bed [149]. For more complex structures, the combination of multiple *in vitro* assays is advised [132]. The pure substance and the vehicle control are suitable references [52].

To establish non-clinical safety in animal studies, manufacturers follow the ICH guidelines [210] as recommended by the US-FDA [54], Commonly, drug disposition is investigated with regards to absorption, distribution, metabolism and excretion [210] using at least two different animal species [211,212].

Unfortunately, in many cases, neither the existing animal models nor the cell-based *in vitro* assays are able to predict the physiopathological responses in humans sufficiently. In addition, most results obtained from *in vitro* assay were inconsistent and require careful interpretation [213]. For this purpose, there is growing interest in alternatives to the usual preclinical settings including quantitative structure-activity relationship models [214,215], toxicogenomics [216,217], or *in silico* approaches to establish *in vitro-in vivo* correlation (IVIVC) or for the purpose of interspecies extrapolation [190].

4.9.4. Clinical evaluation

For some administration routes, the deposition and elimination pathways as well as the pharmacokinetic parameters of the drug are affected by the physicochemical characteristics of nanomedicines including particle size, hydrophobicity or drug release [213]. This applies not only to NBCD but also to some of the medical devices [69].

With regard to the European market, invasively administered nanomedicines, even generics, require an evaluation of clinical safety which is comparable to a new drug application [218]. On the contrary, the generic formulation Lipodox (Sun Pharma, India) was approved by the US-FDA on the basis of bioequivalence studies. To allow assumptions on the *in vivo* performance, pharmacokinetic studies include a determination of the encapsulated, the non-encapsulated and the protein-bound fraction of the API [52] (see Section 4.10.3). For other products addressing the topical or the peroral route of administration, the traditional pathway of biopharmaceutical equivalence provide sufficient evidence to establish efficacy and safety of a generic drug formulation. However, a detailed physicochemical characterization and the evaluation of parameters relevant to clinical safety *in vitro* and *in vivo* is advised [54].

4.10. Biopharmaceutical characterization

The biopharmaceutical characterization of nanomedicines changed our understanding of formulation technology. Traditionally, modifying the release rate enables drug absorption to be controlled by changing the physicochemical features of a dosage form. This paradigm still holds for the vast majority of drug products but, when using nanocarriers, is accompanied by an altered distribution of the drug. Therefore, to establish bioequivalence is a major challenge for the emerging generics market [218]. For most conventional products, manufacturers establish therapeutic equivalence by using the pharmacokinetic parameters of maximum plasma concentration (c_{max}), the time when this concentration is reached (t_{max}) and area under the plasma concentration-time curve (AUC). This applies to pharmaceutical formulations including many of the perorally administered nanopharmaceuticals. However, with regards to NBCD, formulation parameters such as the particle size or the drug release can have major influence on biodistribution. In this context, a detailed evaluation of the deposition mechanisms as well as the identification of quality attributes with a strong influence on the *in vivo* performance is required.

4.10.1. Biorelevant in vitro studies

The *in vitro* drug release is a key indicator of the *in vivo* performance of peroral dosage forms and is generally well-accepted by the pharmaceutical industry [219]. For nanomedicines or nanocarriers, a separation of ultra-fine particles from physiologically relevant liquids is required to enable the measurement of release kinetics [166,169,191]. Thinking about nanocarriers as fragile constructs covered by a protein corona and embeded into a fine network of non-covalent surface interactions [213], the difficulties associated with an efficient separation become more apparent.

To provide a solution for the technical challenges, a number of setups have been evaluated. Kreuter and co-workers tested the drug release from nanoparticles in human plasma using a non-standardized flow-through cell [220,221]. The observed profile revealed a sustained release but there were no appropriate control measurements presented, to investigate the influence of the dialysis membrane on this outcome. Later approaches utilized Franz cells [222], separation by centrifugation [180] as well as dissolution apparatus IV of the United States Pharmacopeia (USP) [223]. A detailed summary of the methodologies applied has been published previously [176]. More often, buffer solutions were used as dissolution media [176].

The valuable of such findings depends on the mechanism of drug release which, among other parameters, can be based on dissolution, dissociation or (to a limited extent) matrix diffusion processes [176]. For some formulations the presence of serum proteins, pH value or the ionic strength are major drivers of drug release. A recent investigation revealed the influence of serum proteins on drug release from microcrystals. Interestingly, the study confirmed a stabilizing effect of the protein corona rather than a direct influence on drug dissolution [224]. Unfortunately, having a closer look at the current literature, only few studies allow the drug release data to be correlated to the *in vivo* performance of nanoformulations (see Section 4.10.2).

Jueneman et al. applied syringe filtration to efficiently separate free fenofibrate from a nanocrystal formulation in biorelevant media [166] and successfully predicted pharmacokinetics in humans [166]. Later findings suggested a limited capability of the method to discriminate slight changes in quality [169,176]. An improved correlation as well as a higher sensitivity was achieved for polymeric nanoparticles using the dispersion releaser technology in combination with a customized *in silico* model [169,176].

For many non-oral dosage forms including nanomedicines, biorelevant conditions are difficult to determine due to the absence of reliable *in vivo* release data. Recent studies utilized imaging techniques [225,226] or applied physiologically-based pharmacokinetic modeling [224] to provide an estimate of the dissolution rate of depot formulations in the subcutaneous tissue. Jablonka et al. determined the *in vivo* dissolution rate of drug nanocrystals in the blood from human pharmacokinetic data [190]. They revealed knowledge gaps arising from the clinical protocol of sample collection and provide convincing evidence of an IVIVC. Improved methodologies would be required in the clinical setting (see Section 4.10.3) to enable a better understanding of the *in vivo* release and support the development of novel biorelevant assays.

4.10.2. In vitro-in vivo correlation

Today, formulation development and quality control of drug products are widely relying on the concept of clinical relevance. Formulation parameters with a high impact on the *in vivo* performance are preferably selected from the physicochemical characteristics. While the quality attributes which correlate to biological performance are generally well understood for conventional dosage forms, for newer technologies, this correlation must be established through biological *in vitro* and *in vivo* studies [123].

At present, most of the cell-based *in vitro* assays barely relect clinical reality. The intercellular and intracellular processes may be involved in the local distribution of the drug and the carrier, but they are often overturned by pharmacokinetic effects. In this context, IVIVC improves our understanding of the clinical reality and essentially contributes to translational research. In return, these findings support the evidence-based development of novel *in vitro* assays.

According to the US-FDA guidance on extended release peroral dosage forms [227] a level A correlation represents a point-to-point relationship between *in vitro* release and the *in vivo* absorption [227]. The predicted plasma concentrations are directly compared to the *in vivo* data. A level B correlation uses the principles of statistical moment analysis by comparing the *in vitro* release data either to the mean residence time or the mean *in vivo* dissolution time [227]. A level C IVIVC establishes a single point relationship between one release parameter and the *in vivo* absorption [227]. These definitions were shaped by the successes in the area of perorally administered drug formulations and cannot be extrapolated to other administration routes.

The drug release rate plays an eminent role in peroral drug delivery and even for many other nanomedicines (*e.g.* for transdermal administration), advanced models reliably predict the *in vivo* performance and have the capability to discriminate between different formulation prototypes [169]. In this context, absorption and exposure can be manipulated, for exampe, by simultaneously reducing c_{max} related toxicity and maintain therapeutically relevant plasma levels.

On the contrary, nanocarrier delivery is characterized by the presence of two distinct fractions of the drug which differ in their distribution and elimination parameters. The *in vivo* conversion between those fractions and, consequently, the release rate determines the ability to control biodistribution and target the API to a certain tissue. Recent approaches utilized physiologically-based pharmacokinetic modeling to identify this *in vivo* conversion (release) rate and to establish an IVIVC for intravenously administered nanocarriers [190]. So far, in the area of nanocarriers, this is one of very few examples providing convincing evidence for a level A relationship and there is more research to be done.

4.10.3. Pharmacokinetics and biodistribution studies

To evaluate the changes in pharmacokinetics and biodistribution of drugs encapsulated into nanomedicines, a preclinical evaluation in animals is required. The safety evaluation follows a strict pattern using at least two different animal species [211,212]. According to recommendations issued by the US-FDA [54], the ICH guidelines on non-clinical safety studies can be applied [210]. They include an *in vivo* characterization of drug disposition with regards to absorption, distribution, metabolism and excretion [210] and so the biodistribution of the drug is widely explored.

Each administration route requires a specific study design. For example, after intravenous injection, nanomedicines with a hydrodynamic diameter below 5.5 nm may undergo renal clearance [228] while larger particles often accumulate in the fine capillaries of lungs, liver, and kidney [229] or can be taken up by the mononuclear phagocytic system of the liver, the spleen and the bone marrow [230,231]. Depending on the specific properties of the parent drug and the nanoparticle system the c_{max} and AUC can increase compared to the conventional formulation [232].

With regards to biodistribution, Mäder and *co*-workers revealed the accumulation of polymeric nanoparticles in the ovaries in mice implying a rather different toxicokinetic profile [233]. Longer circulating times potentially contribute to the distribution of nanoparticles [132,234]. Against this background, the detection of both, the encapsulated and the non-encapsulated form of the drug from the blood plasma may provide more information on the expected tissue distribution pattern of a formulation and support the development of novel *in silico* methods.

5. Conclusion

Today, pharmaceutical companies are facing high barriers to participate in the emerging nanomedicines market. Recent trends in the European regulatory landscape indicate more restrictions and narrow the field of competitors. But a growing knowledge base and a rising number of drug products and medical devices in the market open up new perspectives for the industry.

Evidently, researchers and regulators learned about the risks associated with nanotechnology-related products and developed new technologies to assure their quality and safety. To establish the concept of bioequivalence for nanomedicines is still challenging which affects not only the number of products but also current pricing policies.

Against this background, key players will decide for markets assuring the optimal infrastructure for the production of high-risk nanomedicines in addition to a comparably easy market access. Interestingly, anticipating these developments, China is reducing trade barriers, e.g. by lowering the requirements for the registration of medical devices, and may soon challenge the European and the US medtech industry.

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