Chapter 12
Nanotoxicology and Regulatory Affairs

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Abstract Like other parts of the nanotechnology revolution nanomedicines hold great promise and in the case of nanomedicines the potential for more efficient therapies. Engineered nanomaterials that are used as nanomedicines for therapeutic and diagnostic purposes are often designed to specifically interact with cells of tissues and organs of the human body. However, the unique physicochemical properties of particles at the nanoscale may contribute to adverse effects requiring nanomaterial-specific safety considerations. Therefore, before nanomedicines can be approved by organisations such as the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA) and reach the market, safety, efficiency and efficacy have to be shown. Beginning with some short critical remarks, this chapter addresses the toxicology of nanomaterials referred to as nanotoxicology with special attention to nanomedical applications. The second part of this book chapter will briefly describe the general drug approval process, introduce risk assessment procedures and give an overview of safety and regulatory challenges for nanomedicines.

Keywords Nanotoxicology • Pharmacokinetics • Toxicokinetics • Drug safety • Risk assessment • Nanomedicine regulation

12.1 Introduction

The application of nanotechnology offers many advantages, and products based on engineered nanomaterials (ENMs) are used in nearly all parts of our daily live [1]. Nearly everyone in the developed world have been unintentionally or intentionally in contact with nanotechnology in one way or another, either as consumers, or as workers. Nanomedicines based on nanotechnology and ENMs offer great potential in the treatment of diseases. These new developments promise, for example, improved bioimaging properties and more efficient and targeted drug delivery with fewer side-effects [2]. Due to their small size nanomedicines can cross endothelia

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K.A. Howard et al. (eds.), *Nanomedicine*, Advances in Delivery Science and Technology, DOI 10.1007/978-1-4939-3634-2_12

and epithelia biological barriers and enter organs, tissues and cells [2]. The advantageous properties of nanomedicines include a higher drug dissolution rate and enhanced drug adsorption, and increased bioavailability [2]. As much as ENMs offer the possibility to create new innovative products and new groundbreaking technologies there are, however, concerns that ENMs may pose a threat to human health and also to the environment. These concerns are based on the fact that ENMs may have unique physical, chemical and toxicological properties that differ from the parent material in a way that cannot be predicted by studying the larger-sized material. ENMs are known to be more reactive as their larger-sized counter parts, and it is, therefore, reasonable that ENMs may react with biological systems in new unpredicted ways that could lead to toxicity. Studies on natural occurring (e.g., forest fire) and unintentional (e.g., diesel exhaust) arising particles have shown that particle exposure leads to respiratory and cardiovascular diseases, cancer and possibly allergy in humans [3, 4]. Especially the particle fraction with a diameter less than 100 nm seem to play an important role for cardiovascular mortality and morbidity [3]. Due to the fast growth of nanotechnology and an increasing use of ENMs a whole new scientific branch, nanotoxicology, has emerged.

As nanomedicines consist of ENMs, the concerns regarding their toxicity due to their nano-particulate form lead to safety and regulatory challenges, especially as the field of nanotoxicology is still quite young and not all information that is needed for a comprehensive safety evaluation is available at this point.

12.2 Some Critical Remarks

Although the toxicity of particles in air pollution and their health effects have been investigated for some decades, the field of nanotoxicology with systematic toxicological investigations of ENMs is not more than 10 years old [5]. The term nanotoxicology was first mentioned in the scientific literature in a news article in Science by Robert F. Service in 2003 and was proposed as a new subcategory of toxicology by Donaldson and colleagues in 2004 [5]. Since then, the number of published nanotoxicology related articles has been increased from ~36 in 2004 to ~1919 in 2013. During the last 10 years, it has become clear that the unique physicochemical characteristics of ENMs not only make their toxicity towards biological systems difficult to predict, but that these characteristics also have a profound influence on their behavior in experimental settings. Even small changes of, e.g., their size or surface properties can have large effects on the observed toxicological outcome [6]. Over the years, it has become clear that the toxicological investigations of ENMs require high demands on the characterization of the particles and a well described experimental setting. The results from the first studies within nanotoxicology are hard to interpret due to incomplete or missing particle characterization. In addition, differences in the experimental setting due to studies in different cell line from different tissues and organs, exposure media, concentration and purity of the ENMs, dispersion protocols and media [7, 8] makes it difficult to compare the published

results and to draw definite conclusions on the toxicological potential of ENMs. For example, it became clear that physicochemical properties of ENMs can change considerably in different exposure media and that the results of genotoxicity studies are influenced by the preparation of ENMs, their concentration, used dispersion agents and impurities, cell type used, bioavailability and uptake of the ENMs [7]. These parameters most likely affect the toxicity of ENMs in general.

Today the demands on the researchers and the quality of their investigations are much greater. It is difficult to publish in peer-reviewed scientific journals without a thorough characterization of the used ENMs. But not surprisingly, there is still an apparent lack of consistent results for the toxic effects of ENMs.

Another problem that has been recognized during the past years is the interference of some ENMs with toxicity tests like the Comet assay (metal oxide based ENMs) and the MTT assay (carbon nanotubes) [9–12]. Therefore, appropriate controls are of upmost importance to exclude false positive and negative results. Furthermore, when reading and interpreting the scientific literature, a critical and objective review of the available information should include an assessment if a thorough characterization of the ENMs are made, if appropriate controls are included, and if the studied concentrations are meaningful and cover realistic exposure scenarios. In many studies, animals have been exposed to an unrealistic high dose of ENMs resulting in overload scenarios inducing health effects that are not observed at concentrations that cover realistic worst case scenarios [7, 8].

However, it has to be kept in mind that the vast majority of nanotoxicological studies have been performed in vitro and that there might be a difference to the in vivo situation. One point is that in vitro cell cultures mainly are cancer or transformed cell lines that might react more or less sensitive to ENMs. Another point is that ENMs in in vitro systems interact with and possibly bind to proteins that originate in the uppermost cases from bovine serum, whereas in the in vivo situation, the ENMs are in contact with human blood including all blood cell types as well as the proteins of the human serum. The interaction of ENMs with the different serum proteins and blood cells might influence the results of toxicological studies as well.

12.3 Toxicology of Nanomedicines—Pharmacokinetics and Toxicodynamics

If not directly used as imaging agent e.g., SPIONs or Quantum dots, ENMs can be used as carriers for therapeutic drugs. In either case ENMs are a main component of nanomedicines, which by themselves could pose a potential health threat [6, 13, 14]. Based on studies on the ultrafine nanoscaled particle fraction from air pollution it is known that exposure to these particles increases the risk to develop airway and cardiovascular diseases [13, 15]. Due to increased use and exposure of consumers and workers to ENMs has resulted in concerns about potential adverse health effects of ENMs and the development of a new toxicological field, nanotoxicology. Based on the definition of toxicology by The Society of Toxicology (SOT) [16] nanotoxicology

has been described by Oberdorster et al. “as the study of the adverse effects of engineered nanomaterials (ENMs) on living organisms and the ecosystems, including the prevention and amelioration of such adverse effects” [17]. In comparison to other ENMs, ENMs specifically used in nanomedicines have not been in as much focus of toxicological investigations yet, as the likelihood for an exposure of the general population by nanomedicines is considered to be low. The focus has been mainly on the development of efficient nanomedicines. However, as ENMs are a main component of nanomedicines the general nanotoxicological concepts for ENMs apply and general findings from other ENMs might be transferred to nanomedicines.

The toxicity of ENMs is crucially dependent on their physicochemical characteristics which play, therefore, a key role for the pharmacokinetics and toxicodynamics of nanomedicines. Pharmacokinetics, or toxicokinetics in the case of non-pharmaceutical substances, describes what the organism does with the nanomedicine, whereas, toxicodynamics describes what the nanomedicine does to the organism. Both, pharmacokinetics and toxicodynamics, therefore, reflect the inseparable interconnection of efficacy and toxicity of nanomedicines. This makes the task to develop nanomedicines that have the highest efficacy and, at the same time, the lowest possible toxicity not as simple as it might look at first glance. It is in fact a most difficult challenge as changes of the physicochemical properties can influence the toxicity, absorption, distribution, metabolism, and excretion of nanomedicines at the cellular but also organism level [18, 19]. Therefore, an early implementation of toxicological investigations is fundamental when developing new nanomedicines.

12.3.1 Physicochemical Properties of ENMs that Affect Toxicodynamics

Efficacy and toxicity of nanomedicines are both dependent on the physicochemical properties of the used ENMs. Through changes of their surface charge, shape, size and surface coating, one is able to control and target the drug load and delivery, influence the biodistribution and clearance from the bloodstream. However, a number of toxicological investigations of ENMs have shown that these physicochemical properties also affect the toxic potential of ENMs as they influence their interaction with the organism. The most important physicochemical properties in this connection are size including surface area, agglomeration and aggregation state and porosity; surface chemistry including surface charge and coating; shape; and chemical composition [8, 20–23].

12.3.1.1 Size, Surface Area and Shape

The physical behavior of particles changes dramatically when they reach sizes below 100 nm. Below this size and the smaller the particles are the rules of quantum physics apply more and more resulting in new chemical, mechanical, electrical,

optical and/or superparamagnetic characteristics of the particles. Due to these changes of the characteristics of the particles, it is nearly impossible to extrapolate the biological reactivity and toxicity of ENMs from their larger-sized counterparts [21].

The size is undoubtedly the most important property of ENMs from a toxicological point of view as it influences a number of particle characteristics. These characteristics are high surface to volume ratio, high surface reactivity, absorption of compounds, ability to cross cellular membranes and strong interparticle forces.

How much the reduction of the size to below 100 nm size can change the physicochemical properties of the parent material becomes clear when looking at the example of gold. Gold is known to be one of the least reactive chemical elements. Larger-sized gold particles do not react with oxygen of the air or water. However, when occurring as nanoparticles with less than 10 nm in diameter, gold will burn once it is in contact with oxygen. This example shows that nanoparticles indeed may behave completely different compared to their parent material and this might as well be true for their toxic potential. First of all, the reason for that nanoparticles behave different compared to their parent material lies in the dramatic increase of the surface to volume ratio the smaller the particles become. For example, if a 1 cm cube is divided into 1 million 1 nm cubes the volume is still the same (6 cm^3) but the surface area has dramatically increased from 6 cm^2 to 60,000,000 cm^2. In addition, the percentage of molecules that are at the surface of the particles increases exponentially when the particle size is below 100 nm [13]. This and the enormous increase in the surface area enhances the possibility of ENMs to react with biological systems, including binding to cells, proteins and other biological active molecules. This characteristic of ENMs might, on one hand, be very desirable and useful for their use as nanomedicines but gives, on the other hand, reasons for concern as these interactions might be detrimental as well as uncontrollable.

The size of particles influences where the particles accumulate, how and how fast the body is able to clear particles and presumably directly influence the mechanism and level of toxicity [24]. For titanium dioxide it has been shown that the reduction of their size from 250 nm to 20 nm increases the inflammatory response in lungs of rats and mice; at least when looking at the same mass dose of the particles. At the same surface area, which ultimately means a lesser mass dose for the small particles, the large and small particles induced the same toxicity [13]. Furthermore, the surface area as a direct function of particle size has been shown to be related to inflammation and genotoxicity for nanoscaled carbon black, carbonaceous nanoparticles and titanium dioxide nanoparticles, in vitro and in vivo [25, 26]. For porous or very rough ENMs the specific surface area (Brunauer Emett Teller (BET) surface) should also be considered as this considers all surface molecules not only at the outer surface but also those in pores [21].

If the volume of the particles is kept as a constant, the surface area is dependent on the shape. Spheres have the highest volume to surface ratio, with the ratio decreasing for cubes and fibers. Therefore, the toxicity of ENMs is considered to be dependent on the shape of ENMs [27, 28]. Besides the influence on the surface area, shapes with sharp edges might be prone to faster degradation and in the case of

metal-based ENMs this might be leading to the release of toxic metal ions. However, how important the shape of ENMs is for toxicity is still not clear.

When considering shape and toxicity, nano-fibers are of special concern. The global exposure of workers but also of the general population to asbestos fibers resulted in disorders of the lung and pleura like lung cancer and mesothelioma, pulmonary fibrosis and plaques at a pandemic scale. In 1997 the World Health Organization (WHO) published a definition for the so-called fiber paradigm for high aspect ratio materials [29]. Fibers with a diameter smaller than 3 μm, length greater than 5 μm and an aspect ratio greater than 3:1 are considered as harmful. This is also true for ENMs that have a high aspect ratio. Due to the similarity in their appearance to asbestos and the severity of the asbestos pandemic, high aspect ratio nanomaterials (HARNs) are generally classified as harmful. The mechanism behind the fiber pathogenicity is an incomplete engulfment of the fibers by macrophages also called frustrated phagocytosis. Fibers that are longer than 20 μg in length cannot be fully engulfed and this incomplete uptake process results in pro-inflammatory responses by the macrophages [23]. Chronic exposure to the fibers and subsequent persistent inflammation has been associated with the deposition of scar tissue in the lung (fibrosis), tissue damages and carcinogenicity of high aspect ratio fibers. However, tangled fibers that appear more like a sphere than a fiber will be completely taken up by macrophages and degraded if the material is biodegradable.

One key question, not only for toxicological investigations but also for regulatory purposes is the investigation of the aggregation and agglomeration state of ENMs. Agglomeration is a reversible process as ENM agglomerates are formed by weak bonds whereas aggregates are formed by strong covalent bonds. Aggregation of particles can change profoundly the size and size associated properties like transport, deposition and material release. Therefore, if ENMs are present as aggregates that are within the micrometer range or several hundred nanometers in all directions the material will no longer be considered as a true ENM. Agglomeration and aggregation are of special concern for nanomedicines when applied intravenously directly into the body as this can lead to thrombosis, a potentially lethal obstruction of the blood flow. Agglomeration but eventually also aggregation can occur, e.g., through binding of plasma proteins to ENMs making the surface characteristics and coating of ENMs important factors for the toxicity and intracellular fate of ENMs.

12.3.1.2 Particle Coating and Protein Corona

At the moment ENMs enter the human body they can interact theoretically with any protein of the plasma proteome that consists of ~3700 different proteins. Therefore, it is safe to say that ENMs will never exist as uncoated particles in the body. The interaction between particles and proteins results in a protein corona covering the surface of the particles. Which proteins in particular bind to the ENMs and become a part of the protein corona is, however, dependent on the chemical composition and surface charge of the particles. Several of the proteins in the protein corona of carbon black, silica, titanium dioxide and acrylamide nanoparticles particles have been

identified and a number of these proteins are ligands to receptors at the cell surface [32–34]. Although binding of these proteins to the ENMs might lead to their unwanted uptake, this can also be exploited for targeted drug delivery when the ENMs are deliberately coated with ligands for receptors that are present at the cell surface of the target cells. However, unless the proteins are covalently bound to the surface of ENMs the protein corona is not a static structure and other proteins that are present in the surrounding body fluids can replace the original coating. The coating and the protein corona of ENMs have not only an influence on the clearance and targeting but also on the surface reactivity. If the surface atoms of the ENMs are covered by proteins, their surface reactivity is affected and the biological responses to the ENMs might be reduced [35]. This is exploited when adding a protein or polymer coat to the particles. For example, surface coating or surface functionalization has been shown to improve the therapeutic efficacy and minimizing adverse effects of mesoporous silica nanoparticles based nanocarriers [30]. Furthermore, coating of polystyrene nanoparticles with bovine serume albumin (BSA) has been shown to prolong the circulation time in the blood and significantly reduced the particle clearance compared to uncoated particles of the same size, although ~90 % of the particles were no longer present in the blood after 60 min [31]. However, as it takes approximately 1 min for a blood cell to circulate the body, even small increases of the circulation time will increase the likelihood that nanomedicines can reach their target organ before they are cleared by macrophages. The reason for the quite fast and efficient uptake of uncoated ENMs by macrophages lies in the binding of proteins like immunoglobulin G (IgG) and fibrinogen, so-called opsonins, to the particles. This opsonization of the particles marks the particles for destruction. Opsonins are recognized and bound by macrophages, thereby, enhancing the phagocytosis through these cells.

Taken together, coating of ENMs has several purposes: to avoid agglomeration/aggregation of the particles, to change the surface charge of the particles, target ENMs for uptake by specific cell types, change the bioavailability and degradation of the particles. All this leads to an improvement of the performance of nanomedicines as it diminishes the host defence mechanisms.

12.3.1.3 Surface Charge

Another physicochemical property that has been identified to play an important role for the toxicity of ENMs is their surface charge that can either be positive, negative or neutral. Studies showed that positively charged ENMs induce higher levels of toxicity compared to negatively charged particles of the same chemical composition. The induced toxic responses included cytotoxicity, disruption of cellular membrane integrity, apoptosis, necrosis, loss of mitochondrial membrane potential [36]. The surface charge of ENMs is dependent on their surface coating and surface functionalization. For example can the surface of carbon nanotubes (CNTs) be functionalized using acids. This treatment will result in carboxyl, carbonyl and hydroxyl groups at the surface of the nanotubes thereby leading to a negative charge of the

nanotubes. A negative surface charge has been linked to an increased cytotoxicity [37–42]. On the other hand was shown that a surface functionalization that increases the water solubility of the ENMs decreases the cytotoxicity of CNTs [43].

Interestingly, just by altering the size of ENMs their hydrophobic or hydrophilic properties can change. The reason for that lies in a changed curvature of the particle-water interface. Small-sized ENMs can, therefore, be hydrophobic whereas larger ENMs of the same chemical composition and same coating can be hydrophilic [44].

For the development of nanomedicines, it is important to notice that positively charged particles often form aggregates upon intravenous injection. This can cause, e.g., potentially lethal embolisms in the lung capillaries [45]. The surface charge has also indirectly an influence on the toxicity of ENMs as it influences the efficiency of the uptake of the particles by the cells, the uptake pathways and the cellular distribution [36, 39, 40].

12.3.2 Pharmacokinetics

The physicochemical characteristics of ENMs does not only has an influence on the toxicity of the particles but also on their pharmacokinetics. Pharmacokinetics, the knowledge and investigation of what the body does to a drug, follows the so-called ADME scheme studying the **A**dsorption, **D**istribution, **M**etabolism and **E**xcretion of a drug. Knowledge about pharmacokinetics is, therefore, very important for the development and fine-tuning of the efficacy, but also, toxicity of nanomedicines and plays a crucial role in health risk assessment for nanomedicines.

12.3.2.1 Absorption

In pharmacokinetics absorption is the process by which the drug crosses biological membranes and reaches the bloodstream. The absorption efficiency and involved absorption mechanisms of nanomedicines are greatly depending on how the nanomedicine is administered. Administration of nanomedicines can occur orally, via inhalation, dermally, intravenously, subcutaneously and intramuscularly. Obviously, the absorption process for a drug is bypassed if the drug is directly injected into the bloodstream. As most of the nanotoxicological studies focus on the unintentional exposure of workers and consumers to ENMs, absorption after subcutaneous and intramuscular administration is much less investigated and there is no conclusive information on the absorption process available at this point.

The effectiveness of the absorption process differs greatly with uptake through the skin (dermal) as the least effective and injection, either subcutaneously, intramuscular or intravenously as the most effective administration routes. Although research is still ongoing, most of the studies on dermal absorption of ENMs confirm that the human skin can normally not be penetrated by particles even when they are in the nanoscale range [23]. Neither titanium dioxide nanoparticles nor quantum

dots were able to reach the bloodstream [21]. Even if titanium dioxide nanoparticles were found in the stratum corneum the particles never reached the lower layers of the dermis. The stratum corneum consists of multiple layers of dead keratinized cells, which are difficult to penetrate for ENMs. However, this might be the case if the skin is damaged by wounds, sunburn or skin diseases like eczema. In addition, movement and stretching of the skin, particle charge, follicular openings, gender, and age might affect the barrier function of the skin [21]. But as there are reports showing the absorption or at least penetration of the stratum corneum there is currently no consensus about the ability of ENMs to be absorbed through the skin [46].

Absorption of ENMs via inhalation is probably the best investigated exposure route and will also be discussed in more detail in the section "Pulmonary toxicity". The most important parameter for absorption of ENMs through inhalation is their size and aerodynamic diameter. Depending on these parameters particles will be deposited more or less deep in the respiratory tract. The main mechanism for particle deposition is diffusion due to displacement when the particles collide with the molecules of the air. Depending on the size of the ENMs, they will be deposited in the nasopharyngeal, tracheobronchial or alveolar region [13]. Alveoli are the deepest part of the lung and nanoscale particles have been found to reach this region. The approximate size limits for particle deposition are that particles with an aerodynamic diameter >50 μm do not enter the respiratory tract as they are filtered quite efficiently by the nose, particles >10 μm are deposited in the upper respiratory tract, particles between 2 and 10 μm can reach trachea, bronchi and bronchioles, and particles smaller than 1 μm can reach the alveoli [13]. ENMs that reach the alveoli are mainly cleared by alveolar macrophages [13]. However, if the particles persist in the alveoli they are able to access the pulmonary interstitium either through diffusion or, more likely, transcytosis through the alveolar epithelium. From there the particles can cross the endothelium of the capillary, enter the bloodstream and translocate to systemic sites. Alternatively, the particles could enter sensory nerve endings that are embedded in the airway epithelial, a mechanism that seems to be specific for nanoscaled material [23, 47].

Particles that have been inhaled may be cleared from the lungs via the mucociliary escalator and through this way reach the gastrointestinal tract (GIT) [13, 48]. In addition, the GIT is also an important absorption route for nanomedicines and nanocarriers are currently under development for a more effective oral uptake of drugs and vaccines [46]. Nanomedicines that are administered orally are ingested into the GIT and are absorbed by a process called persorption, the paracellular translocation through transitory leaks in the epithelial cell layer [49–51]. It is thought that loosened tight junctions in the mucosa allow undissolvable particles in nano- but also microscale to be transported in the epithelial cell layer. From there the transport occurs into the sub-epithelial region via the thoracic duct either through lymph tracts or through veins and reach the bloodstream. This process seems to be quite fast as within a few minutes particles are found in the peripheral blood [49–51]. However, newer studies on ENMs showed that the absorption through the GIT increases with decreasing particle sizes and that micro-sized particles are trapped within the Peyer's patches, which are organized lymphoid nodules that are found in the lower regions of the small intestine. Particles trapped there does not seem to

reach the systemic circulation in high numbers [48]. Besides the size, the charge of the ENMs is important as positively charged particles are more efficiently absorbed than negative or neutral charged ENMs [46].

12.3.2.2 Distribution and Cellular Uptake

After absorption or direct administration by intravenous injection the nanomedicine is distributed in the body through the bloodstream. Within pharmacokinetics, distribution is the reversible transfer of drugs away from the site of absorption to other sites within the body, including the target site, into interstitial and intracellular fluids. Again, the physicochemical properties of the ENMs are important for the biodistribution as they influence the way ENMs interact with cells, body fluids and proteins. The binding of ENMs to proteins can influence the mobility of the particles. If these proteins promote cellular uptake of the ENMs in specific organs or immune cells the biodistribution of the particles might be limited.

A general rule is that smaller ENMs have a much greater biodistribution compared to larger ENMs. For example, intravenously administered 10 nm gold nanoparticles were found in liver, spleen, kidney, testis, thymus, heart, lung and brain whereas 50 and 250 nm gold nanoparticles were only found in liver and spleen. One explanation is that the 10 nm particles were too small to be efficiently recognized and internalized by professional phagocytes that normally will clear the blood for foreign particles. Therefore, the 10 nm particles were able to reach more organs compared to the larger particles [52]. Iron oxide nanoparticles with a size of 22 nm were shown to be quickly translocated to the bloodstream and distributed to liver, spleen, kidney and testis after intratracheally instillation of rats [53]. Although research is still ongoing it seems that in general particles with a small diameter (10–20 nm) or a positive charge are more easily translocated through the alveolar barrier of the lung [21]. However, the situation might be different when chronic exposure occurs or in the case of a pathological situation. For example, inflammation seems to increase the translocation of ENMs from the alveoli into the blood and has, thereby, an influence on the biodistribution of the particles [54, 55].

The cell membrane is the last barrier ENMs have to cross if they are used as carriers to transport the drug into the target cells. Due to the particulate or vesicular form of nanomedicines most of the cellular uptake of nanomedicines will occur via active transport mechanisms into the cell. These active transport mechanisms include internalization pathways like phagocytosis, (macro)pinocytosis and receptor-mediated endocytosis via clathrin coated pits or caveolae [21]. Which of these cellular uptake mechanisms apply is greatly dependent on the size of the particles and on their surface coating [21, 56–60]. If particles reach a size of larger then approximately 500 nm they are mainly taken up via phagocytosis by so-called professional phagocytes like neutrophils, monocytes, macrophages, dendritic and mast cells; smaller particles are primarily processed by endocytic pathways. An alternative for the uptake of larger aggregates (0.5–5 μm) might be micropinocytosis [61]. However, at this point the precise role and importance of this pathway for the uptake

of ENMs is not very well investigated. The surface coating can, e.g., allow binding to specific receptors on the cell surface. This is exploited for targeted drug delivery by interaction with receptors that are exclusively expressed at the surface of the target cells. In addition, targeting a specific receptor will also define by which cellular uptake routes the particles enter the cell as some receptors are exclusively found in clathrin-coated pits or caveolae [21]. After entry into the cells ENMs are present in intracellular membrane-coated vesicles. Depending on the uptake pathway, vesicles can be, e.g., endosomes, lysosomes or caveosomes. Furthermore, ENMs were also found in mitochondria, the nucleus or just free in the cytosol [21].

12.3.2.3 Metabolism

Metabolism of drugs covers the biochemical modification or biotransformation of pharmaceutical active substances or xenobiotics, substances that are foreign to the organism. The goal of these biochemical modifications is to convert lipophilic substances into more readily excreted hydrophilic products. The metabolic pathways are the same as for detoxification of poisons and include usually specialized enzymatic systems like the cytochrome P450 oxidase protein family. These enzymes are involved in the first of three metabolic phases where they introduce reactive or polar groups. In phase II, transferase enzymes such as glutathione S-transferases are catalyzing the conjugation of these modified substances to polar compounds that are in some cases further processed in phase III before they are pumped out of the cells by efflux transporters. These reactions are a defense mechanism of the cells to detoxify foreign substances, however, in some cases metabolic intermediates of normally non-toxic compounds can themselves be toxic.

Whereas the metabolism or biotransformation of the pharmaceutical active substance of the nanomedicine is likely to be well investigated and known, the metabolism of their carriers, the ENMs, is generally not very well investigated and understood. So far, ENMs were predominantly found not to be metabolized but that is, of course, very much dependent on the chemical composition of the ENMs. Qdots, e.g., seem to have a very long half-life in the body of several weeks or month. In contrast to nanoparticles, nanoscaled liposomes are likely to be much easier degraded and metabolized if they are able to fuse with cellular membranes. However, generally, there is still very little information available about what happens to ENMs after they have been taken up by cells. And there are concerns that breakdown of the nanostructures can again lead to unique unpredictable molecular responses.

Nevertheless, metabolization of ENMs has to be considered a very important step for clearance of the body from the particles. If ENMs are not metabolized or degraded they might not be excreted and, therefore, accumulate in the cells of the body. This might especially be a problem for repeated long-term administration of nanomedicines. In addition, if the particles are non-biodegradable even a short-term exposure and low toxicity of the administered ENMs might lead to a cumulative toxic effect over time. This is of special concern if an interaction with DNA occurs which could

result in carcinogenesis. Furthermore, it has to be kept in mind that theoretically all biological effects of ENMs can be enhanced if the particles persist within the body for several months, years or even through the entire residual lifetime.

Though, in some cases can the solubility of ENMs result in toxic effects. For example, some or all of the observed toxicity of ENMs consisting of ZnO, CuO or Ag has been attributed to the released metal ions [62, 63]. The dissolution of ENMs can either occur in body fluids but also intracellularly. Here, especially the acidic environment of endosomes and lysosomes are thought to contribute significantly to the degradation of ENMs and to the release of toxic metal ions [64]. However, the pH alone is not in all cases enough for the dissolution of particles. In case of silver nanoparticles the interaction with cellular proteins seem to play an important role for the degradation of the particles as well [65]. Nevertheless, it is important to notice that not all ENMs end up in endosomes and/or lysosomes and that there is a number of materials that either cannot be degraded in endosomes or lysosomes or are able to escape these compartments. Factors that are important for the dissolution rate of ENMs are the size of the particles, roughness, coating, and aggregation state [21, 66–69]. After dissolution of the ENMs the particle compounds might be available for biotransformation and subsequent excretion.

12.3.2.4 Excretion

The two major routes for excretion are through feces and urine and only to a lower degree via the lung and skin. When discussing the excretion of ENMs one has to differentiate between biodegradable and non-biodegradable particles. Biodegradable ENMs are digested and the metabolites excreted by the body through urine or feces, and does no longer pose a health threat. However, excretion of non-biodegradable ENMs might take very long or might even be not at all existing. In general, circulation of the particles in the blood is a prerequisite for their excretion. Also for non-biodegradable ENMs the major routes for excretion are via urine or feces.

For the excretion via urine the blood is filtered in the kidney through the renal glomerula and via this way particles with a size lower than 8 nm can be filtered out of the blood whereas particles that are larger in size will accumulate in the mononuclear phagocyte system (MPS) [21]. The MPS consists of phagocytic cells that are located in reticular connective tissue, which is found around the liver, kidney, spleen, and lymph nodes as well as in bone marrow. In the liver, this system is particularly well developed and the macrophages of the liver, the Kupffer cells, are responsible for clearance of the largest part of the particles. In the case of non-biodegradable ENMs, the particles accumulate and persist in the macrophages. In addition, hepatocytes are able to take up particles via endocytosis but if they can metabolize and secrete the particles into the bile is not known.

Although there is still a great demand for investigations on the fate of non-biodegradable ENMs it seems that particles that are administered intravenously are either rapidly cleared by the kidney or are taken up by the mononuclear phagocyte

system and persist in the body [21]. Water-soluble single-walled carbon nanotubes have been shown to be excreted via the renal route in rats and mice, whereas, titanium dioxide nanoparticles accumulate in the liver and spleen for several weeks [21]. Independent of the physicochemical properties of ENMs, the highest accumulation of particles is in general found in the liver [52, 70].

If particles or agglomerated ENMs reach a size of larger then approximately 500 nm they are mainly cleared from the blood via phagocytosis by so-called professional phagocytes like neutrophils, monocytes, macrophages, dendritic and mast cells. Especially macrophages have been in focus for pharmacokinetics and toxicity studies as they quite efficiently can clear the bloodstream from foreign particles. This might be a great problem for the efficacy of a drug but can also lead to toxicological complications and inflammatory responses if the particles persist within the cells. Positively charged nanomaterials are cleared fast from the blood and their aggregates accumulate in the liver and lung. Neutral ENMs have a decreased rate of uptake by macrophages of the liver or spleen. Neutral surface charge increases, therefore, the half-life of ENMs in the blood and the availability for uptake by other organs. In addition, binding of opsonins leads to enhanced phagocytosis and clearance of the particles from the bloodstream.

12.3.3 Mechanisms of Toxicity of Nanomaterials

Due to the small size of ENMs, the particles enter the organs, tissues and cells of the human body much easier then their larger counterparts. However, one of the most important questions is if the particles induce a toxicological response in the body once they are absorbed and what happens if non-degradable or slowly degradable ENMs accumulate in the body. If talking about toxicity of agents or drugs, a number of terms and definitions are used depending on what is the focus and aim of the toxicological study. Toxicity can be described based on the route, number and duration of exposure, primary toxic effects (target organ), and mechanism of toxicity. Terms like local and systemic toxic effects; acute, subchronic, chronic toxicity; transient, persistent, cumulative, latent toxicity are briefly described in the next section.

12.3.3.1 Toxicity Terms

The toxic effects of a drug, no matter if these are desired therapeutic effects or undesired side effects, can be either local or systemic. Local effects are those harmful effects that occur at the site of the initial contact, e.g., contact dermatitis. Systemic effects occur after absorption of the agent and include toxic effects in organs or tissues that are distant from the side of the original exposure [71]. Local and systemic effects can occur after acute (single) and repeated exposure where the repeated exposure can either be short-term (5 % of lifespan), subchronic (5–20 % of lifespan) or chronic (majority or entire lifespan) [71]. The vast majority of studies on

nanotoxicity are on short-term effects after acute exposure while long-term effects after chronic exposures are mainly unknown. Dependent on when and how long the toxic effects are arising, one distinguishes between transient, persistent and latent toxic effects. Transient effects are temporary and reversible whereas persistent effects are permanent and present during the complete residual lifetime. Latent toxic effects have a delayed onset and can appear days, weeks, month or even years after exposure. Latent toxic effects occur mainly after acute exposure, whereas, cumulative toxic effects are progressing effects after repeated exposure [71].

As nanomedicines are usually a mixture of different components, e.g., the pharmaceutically active drug and the carrier ENM, also other toxicological terms might be important that play a role especially for exposures to mixtures. The toxic effects of the different components of nanomedicines can be *additive* ($2+3=5$; the overall toxic effect is the sum of the toxicity of each component); *antagonistic* ($2+3<5$; at least one of the components antagonize the toxicity of the other); *potentiating* ($0+3>3$; one non-toxic component enhances the toxicity of another toxic component); or *synergistic* ($2+3\gg5$; two toxic components are increasing the overall toxicity much more than the sum of the toxicity of each component). Potentiating and synergistic toxic effects can easily be confused. However, in case of a potentiating toxic effect one of the components has to be non-toxic, in case of a synergistic effect both components have to be toxic.

Although it is not very well studied how the different components of a nanomedicine are affecting each other's toxicity, there are a few examples where the co-exposure with two different kinds of ENMs leads to a potentiating or synergistic toxic effect. For example, pure cobalt and carbide particles have no toxic effect whereas the combination of both components leads to hard metal lung disease caused by the release of reactive oxygen species [72]. Oxidative effects are also observed after co-exposure with carbon black and iron oxide nanoparticles that are not observed for either particle type alone [73].

12.3.3.2 Reactive Oxygen Species, Oxidative Stress and Inflammation

The toxicological effects of ENMs on cells include cytotoxicity and genotoxicity. One if not the most important underlying mechanism for these effects is the induction of oxidative stress in the cells [74, 75]. Oxidative stress is caused by an imbalance between the formation of reactive oxygen species and the antioxidant capacity of the cells [13, 76]. Reactive oxygen species are chemically reactive molecules, and as the name suggests, do contain oxygen. Examples for reactive oxygen species are oxygen itself, superoxide anion, peroxide, hydroxyl radicals and ions, and hydrogen peroxide. These molecules are always present in cells as they are natural byproducts of the oxygen metabolism but, e.g., cellular stress, infection or other environmental factors can lead to an excessive formation of reactive oxygen species. In addition to reactive oxygen species, reactive nitrogen species containing nitric oxide can also be involved in the induction of oxidative stress [77].

There are different mechanisms of how exposure to ENMs might lead to an increased formation of reactive oxygen species. One possibility is the generation of free radicals by ENMs in aqueous suspensions in vitro. Another possibility is an increased production of reactive oxygen species in mitochondria but also the depletion of antioxidants and the subsequent impairment of the antioxidant capacity have been discussed as possible mechanisms. Reactive oxygen species and oxygen-free radicals are mainly produced in the mitochondria and thereby the mitochondria themselves are a major target for oxidative stress and injury.

The existence of too high concentrations of reactive oxygen species within the cell induces lipid peroxidation, mitochondrial damage, damages to DNA, RNA and proteins and lead to the induction of redox sensitive pathways that are involved in pro-inflammatory responses, cell cycle/proliferation as well as apoptosis (programmed and targeted cell death) and necrosis (non-programmed cell death) [74, 75]. The increased formation of reactive oxygen species is thought to be involved in the inactivation of protein functions that are important for cellular DNA repair. Reactive oxygen species might directly attack DNA leading to modified DNA bases like, e.g., 8-oxo-7,8-dihydroguanine (8-oxoG) as the most abundant and best investigated DNA alteration [75, 78]. Impaired repair of DNA alterations such as modified nucleotides is associated with mutagenesis and carcinogenesis. Furthermore, oxidative stress is thought to be involved in a number of different diseases like, e.g., Parkinson's and Alzheimer's disease, and cardiovascular diseases like atherosclerosis and myocardial infarction [74]. Therefore, the increased formation of oxidative stress by most of the investigated ENMs can be linked to a number of diseases and is the reason for concerns about the health effects of ENMs. However, as mentioned before, epidemiological human studies on ENMs are still very rare and many studies are using unrealistic high particle concentrations or purely characterized ENMs. Therefore, definite and especially general conclusions cannot be drawn on the induction of reactive oxygen species by ENMs and the toxicity of ENMs at this point.

12.3.3.3 Genotoxicity and Carcinogenicity

The high surface reactivity of ENMs and the induction of oxidative stress upon exposure to ENMs has raised the concern that they might be genotoxic and carcinogenic. An agent is classified as genotoxic when it has a DNA damaging capacity. Genotoxic events are normally very efficiently repaired by the cellular DNA repair system unless the DNA damage is too extensive. If the latter is the case programmed cell death, apoptosis, is induced. Mutagenesis is the permanent change of the original genetic information and occurs only if the DNA damage leads to persistent mutations within the genome. These persistent mutations can eventually lead to uncontrolled cell growth in form of neoplasms, which in the worst case can be malignant. The formation of malignant neoplasms, also better known as cancer, is called carcinogenesis. Carcinogenesis is a multistep process and requires not only an initiation stage but also a promotion stage. In the initiation stage the cell is exposed to a genotoxic agent whereas in the promotion stage the initiated cell is

exposed to a promoting agent. This multistep process is resulting in carcinogenic characteristics of the cell like evasion of apoptosis, uncontrolled cell growth and metastasis. The promoting agent does not need to be genotoxic itself but either persistent or repeated exposure is required for carconigenesis. Whereas the initiating stage has no threshold (one DNA damaging event is in theory enough at this stage to initiate the cell), the promoting activity of an agent may have a threshold. In principle, the initiating and promoting agent could be the same [71].

The genotoxicity of ENMs and the underlying mechanisms are, at the point of writing this book chapter, not very well understood. In 2012, 4346 articles had been published on nanotoxicology whereof 94 described in vitro and 22 in vivo genotoxicity studies [7]. Although this number has tripled since then, it shows that there still is limited information on the genotoxicity of ENMs available considering the large amount of different types of nanomaterials that has been developed. However, in vitro studies suggest that several ENMs may have genotoxic potential, e.g., carbon nanotubes, C60 fullerenes, titanium dioxide and silver nanoparticles [7, 9, 79–81]. However, the results are somewhat conflicting and often due to limited information on the physicochemical properties of the investigated ENMs or variations in the experimental settings hardly to compare. Despite of these limitations, several mechanisms and factors are currently discussed that could lead to genotoxicity and carcinogenicity of ENMs. These could either be direct primary mechanisms (direct interaction of the particles with the genome), indirect primary mechanisms (interaction with proteins involved in cell cycle, binding to mitotic spindle components, inhibition of antioxidant defense and DNA repair activity or release of toxic ions from soluble ENMs, formation of reactive oxygen species by mitochondria) or secondary mechanisms (formation of reactive oxygen species by inflammatory cells) [7, 82]. These genotoxic mechanisms can result in oxidative modifications of DNA bases, bulky DNA adducts, single and double strand breaks, structural changes of the DNA (deletions, duplications, inversion and translocation of chromosome segments) or changes in the number of chromosomes [7].

Importantly, information on genotoxic and carcinogenic effects of ENMs on humans is even more limited at this point. Epidemiological studies on workers that were exposed to titanium dioxide nanoparticles were inconclusive and could not show an association between exposure to these particles and an increased cancer risk [83]. However, indications for a genotoxic potential of especially non-biodegradable persistent ENMs should of course be taken seriously and require further and more detailed investigations.

12.3.3.4 Neurotoxicity

Neurotoxicological health effects are adverse effects on the brain and the central nervous system. Normally, the blood-brain barrier protects the brain from entry of foreign particles or other unwanted compounds. The passage even of small molecules is tightly regulated and efficient translocation of drugs through the blood-brain barrier is hard to achieve. However, depending on their physicochemical

properties, especially their size and surface charge, nanoscale particles might be able to enter the brain or the central nervous system. ENMs that are 20–50 nm in size as well as hydrophobic particles might be able to enter the brain even if the blood-brain barrier is intact [23, 84–86]. However, the reports in this matter are conflicting and intravenous injection of ENMs like 40 nm gold nanoparticles did not lead to translocation of a detectable amount of particles into the brain [87]. In contrast, a recent study by Huang et al. showed an accumulation of intravenously administered lipid nanoparticles in the brain parenchyma of mice after 3 h. The particles persisted there for more than 24 weeks [88]. Furthermore, for polymeric nanoparticles the surfactants seem to be more important than the size of the particles [89]. Aging, injury or disease may limit the protective capacity of the blood-brain barrier and allow for an easier access [90]. Another possibility is the entry via the olfactory bulb where there is a connection between the nasal epithelium and olfactory neurons [47]. This has been shown for carbon nanotubes, gold nanoparticles, quantum dots and manganese oxide nanoparticles [13, 47, 91–94]. ENMs were found in the olfactory bulb but have also been found in the hippocampus [95]. The entry into the brain was shown to be associated with an inflammatory response [93, 95, 96]. Although animal studies have shown that ENMs can reach the brain through the olfactory bulb, it is not known which role this entry pathway might occur in humans, as humans have a significantly less developed olfactory bulb compared to rodents [21]. In addition, it is not known what the health effects of ENMs actually are after they reach the brain or central nervous system. Animal and in vitro studies suggest that the presence of ENMs in the brain can cause brain damage. Neutrophils and lymphocyte numbers as well as protein carbonyl levels were increased and oxidative stress, lipid peroxidation and inflammatory responses (glia activation) have been discussed to be induced as a result of the high surface area and reactivity of ENMs [47, 76, 88, 93, 97, 98]. In addition, it is more likely that neurotoxicity is of chronic nature than to be acute due to the difficulties of reaching the brain [90].

12.3.3.5 Pulmonary Toxicity

From studies on nanoscale particles in air pollution we know that their inhalation can have adverse health effects and is associated with increased risk to develop cardiovascular diseases, lung fibrosis and lung cancer. The adverse health effects that are associated with inhalation of particles are occurring due to the deposition of the particles in the lung. Although there has been a number of studies on nanoscale particles in air pollution on human health, there are still only limited data available on the health effects of ENMs. This is also due to the difficulties to separate exposure to ENMs from background exposure of ambient particles. In a study on workers that were exposed to polyacrylate nanoparticles it has been suggested that these particles induce pleural effusion, pulmonary fibrosis and granuloma [99]. The respiratory effects that have been described are mainly inflammation, oxidative stress and functional disturbances. The inflammatory response includes local invasion of

leukocytes and release of cytokines [21]. As described before, the toxic effect of ENMs are dependent on the size and shape of the particles. Nanoscale particles have been shown to reach the alveoli, the deepest part of the lung. Alveoli have an extreme large surface area (estimates are between 30 and 100 m^2) but the distance between the surface of the alveoli and the bloodstream measures only 2 μm [21]. Therefore, this region is less protected against inhaled particles [100]. Macrophages are mainly responsible for clearance of particles in the lung via phagocytosis. However, the efficiency of this process is strongly dependent on the size of the particles [21, 101]. It seems that alveolar macrophages are unable to recognize particles as foreign and to phagocyte them when they are less than 70 nm [23]. In contrast, nanofibres with a length of more than 20 μm are too long for phagocytosis. In both cases, the particles are suspected to stay in the lung for month or even years resulting in nonspecific pulmonary inflammatory responses. These inflammatory processes might even spread systemically as, e.g., also an increased risk for cardiovascular diseases is associated with pulmonary exposure to ENMs [23].

12.3.3.6 Cardiovascular Toxicity

Cardiovascular toxicity of ENMs or other nanoscale particles, e.g., ultrafine particles in air pollution, has mainly been observed after exposure via inhalation. Several epidemiological studies have shown the association between particles in air pollution, especially the ultrafine fraction, and cardiovascular diseases [102]. The observed short-term health effects after exposure to nanoscale particles and particles in air pollution include arrhythmia, coagulation disturbances, thrombosis, blood pressure abnormalities and in the long perspective a generally increased risk for development of cardiovascular diseases. Although the reasons and mechanisms are still somewhat unclear, it is thought that deposited particles in the lung induce inflammatory responses and conditions that cause these health effects. The release of inflammatory and prothrombotic mediators from the site of exposure into the blood might cause the activation of immune cells leading to the development of these adverse conditions [103]. Especially if these inflammatory responses are chronic and become systemic they will, obviously, have stronger effects on the cardiovascular system. After uptake by alveolar macrophages ENMs might be translocated from the respiratory to the cardiovascular system where the particles can directly induce cardiovascular toxicity by induction of inflammatory responses through cellular stress and increased release of reactive oxygen species [21]. Although epidemiological data for ENMs are still rare, it is believed that ENMs underlie the same toxicological mechanisms and, thereby, have somewhat the same adverse effects on the cardiovascular system as ultrafine particles in air pollution. As several studies have shown that ENMs can induce pulmonary toxicity this is believed to be an indicator for potential cardiovascular damage due to the close association of pulmonary and cardiovascular toxicity [104].

12.3.3.7 Reproductive Toxicity

The reproductive toxicity, which includes adverse effects on the sexual function and the fertility of adult males and females as well as the developmental toxicity in the offspring, is probably the least investigated toxicological effects of ENMs. Some studies in mice have shown that titanium dioxide nanoparticles were able to cross the blood-testes barrier and reduced the sperm production in the offspring of the treated mice. In addition, it has been suggested that these particles might be able to affect the development of the central nervous system in the offspring as they affected the gene expression of genes involved in the development and function of the neural system [46]. However, since a possible accumulation of ENMs has been found, further studies are needed to exclude any reproductive and developmental effects due to an exposure to ENMs or nanomedicines. In addition, the potential of ENMs to cross the fetal-placental barrier has to be investigated as well [46].

12.4 Drug Safety Testing

Like conventional drugs, also nanomedicines have to be approved before used on patients. In the U.S.A. this is done by the U.S. Food and Drug Administration (FDA) and in countries of the European Union, the European Medicines Agency (EMA) is the responsible regulatory agency. The approval process involves several phases including preclinical studies as well as clinical trial phases. In these different phases the safety and efficacy of the drug is investigated by the applicant under the supervision of the responsible regulatory agency.

12.4.1 Preclinical Studies

Before entering the clinical trials drugs are tested in pre-clinical studies, normally by the drug-developing pharmaceutical company. The aim of these pre-clinical studies is to collect basic safety and efficacy data. Based on these data a plan for further testing of the drug on humans is developed and an application for clinical trials is submitted. Another important goal of these studies is to ensure that the drug is a promising candidate that justifies the enormous costs and efforts that are associated with a drug approval process. Therefore, these pre-clinical studies are quite extensive and include in vitro cell culture studies as well as in vivo animal studies to investigate the preliminary efficacy, toxicity and pharmacokinetics of the drug. In the case of nanomedicines, nanotoxicological aspects have to be considered in addition to standard toxicological investigations and evaluations.

12.4.2 Clinical Trials

The clinical trials involve three phases where the drug is tested on either healthy volunteers (phase I) or patients (phase II and III). The goal of phase I is the determination of the most frequent side effects of the drug and, frequently, the pharmacokinetics of the drug that gives information about how the drug is taken up, transported, metabolized and excreted. Typically, between 20 and 80 volunteers are involved in phase I and the safety of the drug is stressed. However, there are circumstances when patients have to be enrolled. This is the case when the drug is expected to cause severe side-effects in healthy individuals. After a successful phase I, approximately 100–300 patients are enrolled in phase II where the effectiveness of the drug is investigated. Normally, the effect of the drug on patients will be compared to patients receiving either a placebo or standard treatment. Phase III contain 1000 or more patients to further investigate the safety and effectiveness of the drug. Different dosages and the use of the drug in combination with other drugs are studied. Based on the results from the clinical trials the authorities decide whether the drug can be approved or not. However, as it is not possible to predict and determine all side-effects and especially long-term effects in the clinical trials, the drug will be further monitored to detect any adverse effects when on the market. Again, when investigating the safety of nanomedicines their specific nano-related characteristics and properties have to be taken into consideration in all phases of the drug approval process as special nanoscale related safety issues have to be addressed.

12.5 Risk Assessment of Engineered Nanomaterials

12.5.1 Risk Assessment

Toxicological investigations of ENMs are important not only for drug safety testing, but are also essential parts of the risk assessment of these agents. Nanomedicines and the used ENMs have to be manufactured and depending on the scale of this manufacturing process unintentional exposure of workers could occur. Risk assessment is also necessary to regulate the use of nanomedicines and ENMs properly. To cover all aspects of safety considerations for nanomedicines a short introduction into risk assessment will be given based on the WHO tool kit and IPCS harmonization project (WHO Human Health Risk Assessment Toolkit: Chemical Hazards; http://www.who.int/ipcs/methods/harmonization/areas/ra_toolkit/en/).

For the investigation of the adverse effects of a newly developed drug, a broad spectrum of methodologies are used that range from experimental in-vitro studies to animal studies and epidemiological investigations on whole populations. The identification of adverse effects form the basis for risk assessments of any given chemical,

physical or biological agent. *Risk Assessment* is a process where it is evaluated to which degree, and with which probability, these agents affect human health and the environment. It is the primary objective to identify and characterize potential hazards, estimate exposure and assess the overall risks for humans or the environment. The assessment if an agent poses a risk is the first component of a risk analysis that also includes risk management and risk communication.

When talking about risk it is important to keep in mind that a hazardous substance does only pose a risk to humans but also the environment if there is a likelihood for an exposure. Risk is therefore defined as a function of hazard and exposure:

$$\text{Risk} = \text{f}\left(\text{Hazard};\ \text{Exposure}\right) \tag{12.1}$$

In Eq. (12.1) is risk = zero if either hazard or exposure equals zero. Equation (12.1) is probably the most important risk assessment paradigm and can be very illustratively explained using the tiger in a cage example. Everybody will probably agree that it is a risk to visit a living tiger inside its cage, especially a hungry one. In this situation, we are exposed to a hazardous biological agent. However, when the tiger is separated from the visitor by a cage there is no risk to the visitor (there is a hazard but no exposure) just as a mounted tiger outside a cage poses no risk to the visitor (there is an exposure but no hazard). This example shows that for a thorough risk assessment the exposure assessment is just as important as the identification of the potential hazards of chemical, physical or biological agents.

A risk assessment will always begin with a problem formulation to establish the scope and objective of the risk assessment. The risk assessment itself consists then of four steps including *hazard identification*, *hazard characterization*, *exposure assessment* and *risk characterization.* In the following chapters a short overview over these four steps is given.

12.5.1.1 Hazard Identification

The first step in risk assessment is the hazard identification that is mainly based on the results from toxicological studies. These toxicological studies include human studies (mostly epidemiological studies), animal-based and in vitro toxicology studies as well as structure-activity studies. Although risk assessment and toxicology also include the investigation of adverse effects on the environment, the following will focus on the health hazards and effects on humans as nanomedicines are primarily intended to be used in humans being well aware of that the production of nanomedicines could pose environmental hazards and risks.

The purpose of the hazard identification is to identify (1) the specific hazard, (2) the type and nature this hazard may have to an individual or (sub)population and (3) investigate if exposure to the agent has the potential to be harmful.

The hazard identification begins with the identification of the chemical composition and, as nanomedicines consists of nanomaterials, particle characteristics of the nanomedicine. Identifying the chemical composition of a nanomedicine will give

information if the parent material is already classified by the CLP regulation of the European Union (CLP stands for "Classification, Labelling and Packaging") and if it is already known to be hazardous. The health hazards that are CLP classified include acute toxicity, sensitization of respiratory tract and skin, skin corrosion and irritation, serious eye irritation and eye damage, reproductive toxicity, germ cell mutagenicity, carcinogenicity, specific target organ toxicity after single and repeated exposure, and aspiration toxicity.

In addition, as previously mentioned high aspect ratio nanomaterials like, e.g., nanotubes, nanofibers, nanowires and nanorods are generally considered hazardous when they are at the same time biopersistent, able to pass ciliated airways and able to initiate frustrated phagocytosis, which leads to the release of pro-inflammatory molecules.

If the nanomaterial is not categorized as high aspect ratio nanomaterials (HARN) there has to be investigated if the nanomaterial induces acute or chronic toxicity including genotoxicity, neurotoxicity, carcinogenicity, pulmonary, cardiovascular, or reproductive toxicity or if the nanomaterial accumulates in organs. If there cannot be excluded that the nanomaterial is potentially hazardous one proceeds with the hazard characterization.

12.5.1.2 Hazard Characterization

Whereas the hazard identification recognizes the type and nature of the hazard, the objective of the hazard characterization is to obtain a qualitative or quantitative description of the inherent properties of the agent that is potentially hazardous when one is exposed to it. A quantitative description will, wherever possible, include a dose-response assessment, identification a no-observed-adverse-effect level (NOAEL), no-observed-effect level (NOEL) or cancer potency factor and take uncertainty factors into account. In addition, based on dose-response assessments no effect levels (NEL) of an agent are derived.

The information on NOAEL and an eventual cancer potency factor are used to establish tolerable daily intake (TDI), acceptable daily intake (ADI) value as guidance values while including uncertainty factors like, e.g., interspecies and intraspecies variability, and data quality. TDI and ADI are both referring to a dose that is safe to consume for humans during an entire lifetime. ADI is used for, e.g., food additives, whereas, TDI is used for agents we are unintentionally exposed to like, e.g., air pollution, contaminants of water.

Depending on the uncertainty level of these data, e.g., if there has to be extrapolated from in vitro or animal studies to humans, if it is necessary to include susceptible population groups etc., uncertainty factors in the range of 10–10,000 are applied to cover also worst case scenarios and population groups. By applying an uncertainty factor the acceptable concentration for the exposure to an agent is reduced to a value where also the most susceptible population groups are not experiencing adverse health effects. Thereby it is avoided that parts of the human population might be unprotected.

Like for the hazard identification, data are obtained from human studies (mostly epidemiological studies), are animal-based or in vitro toxicology studies as well as structure-activity studies or combinations of these studies.

12.5.1.3 Exposure Assessment

The exposure assessment does not only include the investigation whether there is a contact with a potentially toxic agent. It determines also the concentration, route and duration of exposure. It has also the goal to establish safety margins and thresholds by evaluating the likelihood and level of exposure. An exhaustive exposure assessment requires that all possible exposure scenarios are taken into account and that includes the identification of particular susceptible population groups like children, pregnant woman, elderly and predisposed people. Another important part of the exposure assessment is the identification of the route and duration of exposure. In addition, this knowledge is important for the regulation and legislation of toxic agents but also nanomedicines. As described before, the exposure can occur orally, via inhalation, dermally, intravenously, subcutaneously and intramuscularly and the toxicity of a substance may be dependent on the route of exposure. For the estimation of exposures, either measurement or modelling approaches are used. In most cases when unintentional exposures occur the exact measurement and determination of an exposure is not available and a worst case scenario is modelled.

For nanomedicines the administered dose is exactly known. However, the internal dose is dependent on the absorption and excretion of a drug and can therefore vary from the administered dose. In some cases, the internal dose can be estimated using biomarkers. Biomarkers are measurable indicators for the presence of a substance in the body and can be measured in tissues or body fluids like blood, urine but also feces. The duration of an exposure can be acute, subacute, subchronic or chronic. The acute exposure has a duration of less than 24 h and is often a single exposure. Subacute exposure refers to repeated exposures with a duration of up to a month and subchronic exposure lasts for 1–3 month. If the exposure duration exceeds 3 month chronic exposure occurs.

12.5.1.4 Risk Characterization

The last step in the risk assessment process is the risk characterization. The aim of the risk characterization is, if possible, quantitative determination of the probability that known potential adverse health effects occur under defined exposure conditions. These exposure conditions might be actual or predicted exposures. Risk characterization includes the results that have been obtained from hazard identification and characterization and exposure assessment. Based on these results, risk quotients or margins of safety are calculated and exposure and no effect levels are compared to estimate the risks. However, there are no

absolute measures of risks and the conclusion if and when a given agent comprises a risk might vary from scientist to scientist especially if they include implicit value judgments.

12.6 Regulatory Affairs

The regulation of the use of ENMs is still debated due to the relatively short time period ENMs have been in focus of toxicological investigations. Many toxicity and safety related uncertainties of ENMs have not been clarified and this gap in knowledge results of course in uncertainties about the safety of nanomedicines. Nevertheless, nanomedicines have been authorized by licensing agencies like the FDA and EMA for more than 30 years.

The primary regulatory bodies in the U.S. and European Union (EU) that are relevant for the regulation of nanomedicines are the FDA and EMA, respectively. The EMA is accompanied by several committees and groups, whereof, the Committee for Medicinal Products for Human Use (CHMP), the Innovation Task Force (ITF) and the New and Emerging Technologies (N&ET) Working group are relevant for the regulation of nanomedicine. In the case of the FDA the following centers and groups are relevant: Center for Drug Evaluation and Research (CDER), Center for Devices and Radiological Health (CDRH), Center for Biologics Evaluation and Research (NTIG) and Nanotechnology Task Force (NTF). In other countries, national agencies are responsible for the approval of nanomedicines.

12.6.1 Definition of Engineered Nanomaterials for Regulatory Purposes

For risk assessments and regulatory purposes a definition of the term nanomaterial is of utmost importance. However, the sheer number of different nanomaterials makes a definition much more complex than one perhaps first realizes. On one hand, the definition for nanomaterials has to be so comprehensive that it includes all nanomaterials but should, on the other hand, be also be simple and precise as possible. At the moment, there are no standardized definitions what a nanomaterial is and the definition varies between organizations and countries. One of these definitions was proposed by the International Organization for Standardization (ISO) in cooperation with the European Committee for Standardization (CEN). According to the ISO/TR 11360:2010 definition a nanomaterial is a material with any external dimension in the nanoscale or having internal or surface structure in the nanoscale with nanoscale (or nano range) defined as size range from approximately 1 nm to 100 nm (ISO/TR 11360:2010 Nanotechnologies—Methodology for the classification and categorization of nanomaterials https://www.iso.org/obp/ui/#iso:std:iso:tr:11360:ed-1:v1:en).

From a scientific point of view an implementation of a fixed size limit might not make sense and the approximate size range might be preferred. However, for regulatory purposes a fixed size limit is needed and, therefore, implemented by the EU Commission. This definition is based on the ISO definition, an opinion of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and a report of the Joint Research Centre (JCR). 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 1–100 nm.

Fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

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 %". In addition, the European Commission has acknowledged that a upper limit of 100 nm might not always be scientifically justified and that there are special circumstances prevailing in the pharmaceutical sector (EU Scientific Committee on Emerging and Newly Identified Health Risks. Scientific basis for the definition of the term 'Nanomaterial'. European Commission, Brussels, Belgium (2010)).

In the U.S.A. the FDA has another definition for nanomaterials and according to this a nanomaterial is defined to be any material with at least one dimension smaller than 1000 nm and a nanoparticle is an object with all three external dimensions in the size range from ~1 nm to 100 nm (http://www.fda.gov/RegulatoryInformation/Guidances/ucm257698.htm; accessed August 2014).

The lack of an adequate definition of nanomaterials becomes especially problematic when dealing with follow-up nanomedicines that are based on already approved medicines formerly not classified as nanomaterials or not registered to contain nanomaterials.

Another reason for the still ongoing debate on the definition of nanomaterials is the challenge of a comprehensive characterization of ENMs used as nanomedicines. The methods that are available for characterization are not necessarily applicable for ENMs in complex mixtures and sometimes only the primary material might be suitable for characterization. A too rigid definition and regulation might, therefore, lead to the reluctance of regulatory agencies to issue manufacturing licenses or marketing authorizations. Therefore, not only a definition for nanomaterials is needed but also a definition of standards for the characterization of nanomaterials [105].

12.6.2 Regulation of Nanomedicines

The exact definition of a nanomaterial is only one of many questions that have to be addressed for a proper regulative approach for nanomedicines. In addition, the situation might be even more complicated when it has to be decided if a nanomedicine that uses ENMs as carrier is a medicine (medical product) or a medical device? This

classification is not unimportant as medical products and medical devises are regulated in different ways. Furthermore, before a new nanomedicine can be accepted for the use on patients it has to be decided, which regulatory regime is applicable. A medical device fulfills its function by physical means like mechanical or chemical action, whereas, a medical product exclusively fulfills its function by pharmacological, immunological or metabolic means [105]. Clarification on these matters is of course of uppermost importance not only for the safety of patients but also for the pharmaceutical industry that demands a greater harmonization in current nanomedicine regulatory framework. In the EU, the decision on the classification as a medical device or medicine is based on the EU Directive 2001/83/EC on human medicines, as amended, and the EU Directive 93/42/EEC on medical devices, as amended. According to these directives the decision is been made according to the principal mode of action of the nanomedicine. Especially for nanomedicines, which have a complex mode of action, this may prove difficult as their mode of action might involve and combine physicochemical and pharmacological properties. In addition, in some situations, when the nanomedicines are based on viable cells or tissues, they might also be classified as advanced therapy medicinal products and fall under the Regulation (EC) 1394/2007 on advanced therapy medicinal products [105].

Toxicological investigations are the basis for the safety and risk assessment of nanomedicines and as such crucial for their approval. For conventional medicines a battery of OECD approved methods are available for the investigation of eventual toxic effects of a drug. However, it is not clear if these tests are applicable for ENM based nanomedicines. For example, bacteria-based genotoxicity assays like the Ames test may not be appropriate as ENMs may not be able to penetrate the bacteria [105].

Taken together, it becomes clear that the regulation of ENMs and nanomedicines is still under development. Just recently as from 21 June to 13 September 2013 the European Commission had launched a public consultation on the modification of the REACH Annexes (REACH—Registration, Evaluation, Authorisation and Restriction of Chemical substances) on nanomaterials with the aim to improve the clarity on how nanomaterials are defined and their safety demonstrated. This consultation was open for the public and interested stakeholders. The European Commission states that “The REACH legislation must ensure a high level of health, safety and environmental protection. At the same time it should permit access to innovative products and promote innovation and competitiveness.” (http://ec.europa.eu/nanotechnology/policies_en.html).

12.7 Conclusion

Efficacy and toxicity of nanomedicines are inseparable interconnected as changes of the physicochemical properties can influence absorption, distribution, metabolism, and excretion of nanomedicines at the cellular but also organism level. For example, lipid particles or biodegradable ENMs might be less harmful than non-

degradable inorganic ENMs and are most likely to be used in the clinic sooner. Therefore, an early implementation of toxicological investigations is fundamental when developing new nanomedicines.

Although there are no nano-specific directives and regulations at this time, it is important to point out that nanomedical products are not unregulated. Although it is not clear at this point if this procedure is adequate, the FDA and EMA are applying of course the existing legislation on medical products and devices, tissue engineering etc. that are relevant for nanomedicines. Despite the doubts, in 2013 as much as 247 nanomedicine products were listed in FDA registers as approved or to be in various stages of clinical trials most of them attended to be administered intravenously [106]. Hopefully, in time the safety and regulatory challenges that come with nanomedicines are solved to utilize the full potential of nanomedicines.

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