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Upconverting Nanoparticles: Assessing the Toxicity

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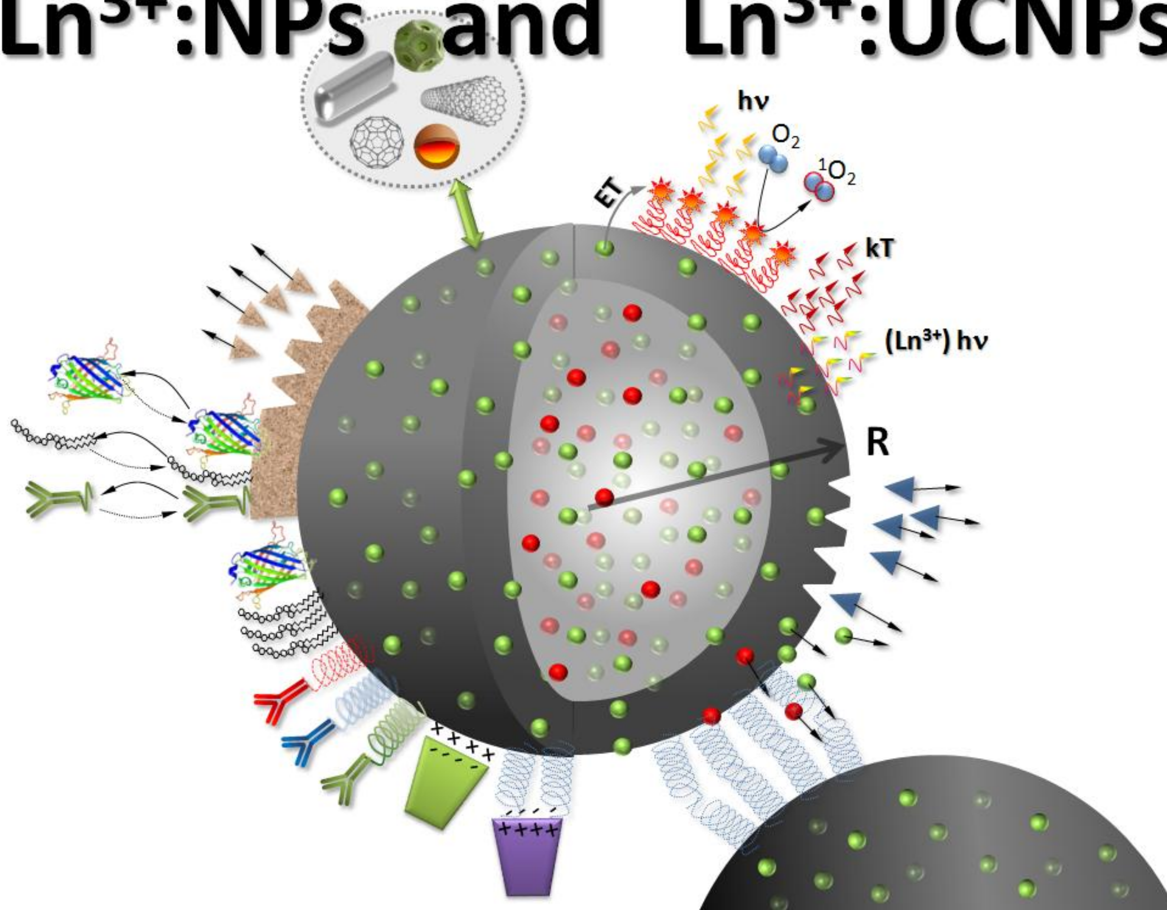

Based on survey of existing studies, low nanotoxicity of lanthanide doped upconverting nanoparticles holds promise for their safety and suitability for biomedical detection and imaging.

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REVIEW ARTICLE

Upconverting Nanoparticles: Assessing the Toxicity

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Lanthanide doped nanoparticles (Ln:NPs) hold promise as novel luminescent probes for numerous applications in nanobiophotonics. Despite excellent photostability, narrowband emission, efficient anti-Stokes emission and long luminescence lifetimes, which respectively respond to the requirements for prolonged observations times, multiplexed and background free detection, concerns about their toxicity is still an issue for both *in vivo* or *in vitro* applications. Similarly to other chemicals or pharmaceuticals, the very same properties that are desirable and potentially useful from a biomedical perspective, can also give rise to unexpected and hazardous toxicities. In engineered bionanomaterials, the potentially harmful effects may originate not only from their chemical composition but also from their small size. The latter enables the nanoparticles to bypass the biological barriers, thus allowing deep tissue penetration and the accumulation of the nanoparticles in a number of organs. In addition, nanoparticles are known to possess high surface chemical reactivity as well as large surface-to-volume ratio, which may seriously affect their biocompatibility. Herein we survey the underlying mechanisms of nanotoxicity and provide an overview on the nanotoxicity of lanthanides and of upconverting nanoparticles.

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1. Introduction

Nanoscience and nanotechnology have been driven by both fundamental and technological interests in an effort to develop new "miracle" materials for a broad range of applications. In fact, the ability to design and manipulate materials at near atomic level has opened exciting avenues for the design and synthesis of materials with physical and chemical properties that are unique and constitute the basis for the plethora of potential applications. Nevertheless, the increase in the production and the breadth of potential utilization of nanoparticles requires an assessment of the consequences and impact on human health, wildlife and the environment.

With possible exception of some forms of carbon-based nanoparticles arising from combustion of organic matter exposure of humans to nanosized materials has not been significant. However, over the last decades of the 20th century the production of many types of nanomaterials has grown exponentially and a large number have found commercial uses, particularly metal oxides (*i.e.* titanium dioxide in sunscreens or as antibacterial coating additive).

Numerous and extensive studies concerning various aspects of biomedical use of nanomaterials are published every year. These studies describe in detail toxicology [1-10], clearance [2, 11-13] or biodistribution [1, 2, 8, 10, 13-30] of various nanomaterials (such as gold nanoparticles, carbon nanoparticles and nanotubes, quantum dots, magnetic nanoparticles *etc.*). At the same time an insufficient amount of data is available to the community on the toxicity of lanthanide doped nanoparticles to properly assess their impact on human health. Thus, systematic research on these key materials that show great promise in biomedicine must be carried out in order to arrive at conclusions with respect to their toxicity.

In this paper we review the nanotoxicological studies that have been reported and potential health hazards of using fluorescent upconverting nanoparticles (UCNPs) doped with lanthanide ions.

2. General remarks on nanomaterials toxicity

Currently, knowledge of nanoparticles toxicity is drawn from studies on nanoparticles such as metal oxides, carbon, silica and combustion products. Due to paucity of data it is impossible to provide a generalized conclusion, however several important facts have been observed.

Most importantly, at the nanoscale, which goes between 1 and 100 nm (Figure 1) and contains approximately 10 to 10,000 atoms, the materials demonstrate properties different from the bulk, with all the associated chemical and physical properties. Thus, for material of the same chemical composition but of different size, a multitude of chemistries and physical properties are expected where every size stands different. Due to their size nanoscale materials possess large surface areas, usually in the 100-1000 $m^2 g^{-1}$ range. At the nanometer scale the surface is an important parameter, which cannot be ignored since the majority of the atoms are located there. In addition, atoms at the surface usually possess fewer nearest neighbours or co-ordination numbers thus they have dangling (i.e.unsaturated) bonds that are exposed at the surface and carry a partial charge, which increases the energy of the surface. The systems preference will be to reduce the free energy by enforcing the reaction on the dangling bonds thus reducing the surface energy. The surface energy may also be reduced through surface relaxation and surface restructuring. Thus, surface functionalization, grafting, adsorption, homo and hetero agglomeration and reactivity as well as the way nanomaterials interact with their environment and with others of its kind are all affected by the size and surface energy. Notwithstanding, size and surface energy, the shape of nanomaterials is known to have a profound effect on the size dependent properties [31, 32]. We can cite the well-known case of spherical and rod-like gold nanoparticles, which show very different colors, due to the single, and two frequencies (transversal and longitudinal) of oscillation of the electrons respectively.

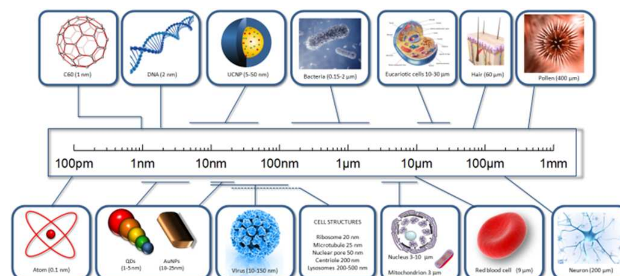

Fig. 1 The size of lanthanide doped NPs referenced to other, biologically relevant compounds, cells and organisms.(pictures reprinted from: (C60) ©Leonid_Andronov/fotolia, (DNA) ©ag_visuell/fotolia, (bacteria) ©nobeastsofierce/fotolia, (hair, neuron) ©Sebastian_Kaulitzki/fotolia, (pollen) ©abhijith3747/fotolia, (virus) ©abhijith3747/fotolia)

The challenge of assessing the impact of nanomaterials on our health and the environment is not straightforward. There are a plethora of other features in addition to the fundamental parameters mentioned above, which may act alone or in concert such as: chemical composition, purity, doping, hydrodynamic size, morphology, size heterogeneity, redox properties, tendency for aggregation, nature and composition of the shell or coating material, surface modifications and surfactants, chemical and colloidal stability, solubility, biodegradability, concentration, duration of exposure and unknown interactions with other engineered nanoparticles (QDs, AuNPs, CNT, DWCNT *etc.*) or behavior under electromagnetic field exposure (*e.g.* heating, photoactivation *etc.*) [19, 23].

A good example of problems associated with nanotoxicology as compared to conventional toxicology relates to TiO_2 nanoparticles (NPs), used commonly as UV absorber and protection against skin cancer in sunscreens. Although these engineered nanoparticles have been studied extensively and are well known, there are still significant knowledge gaps and ethical issues related to their use [33]. For instance, 48 h experiments on Zebrafish, the nano-sized TiO_2 were almost non-toxic from chemical perspective, while some alterations in the expression of genes upon long-term exposure were found [33, 34]. The TiO_2 NPs efficiently absorb UV light, but their photocatalytic properties (generation of radicals on their surface) under high energy photons irradiation or superhydrophilicity are usually neglected.

The nanotoxicity studies are usually performed as classical toxicology studies, *i.e.* only chemical composition and substance dose (in mg per kg of body weight) are taken into consideration, "sterile" laboratory conditions are assumed and the tests are performed on test animals, such as rats, mice and hamsters. However, there are numerous unexpected properties of nanoparticles, resulting solely from dimension change (owing to *e.g.* conditions of synthesis, crystallite size or aggregation of nanoparticles into larger structures), which cannot be predicted and reliably measured during such analysis. Therefore, the results of classical toxicity tests, obtained to date, can be questionable and may lead to unjustified conclusions and groundless extrapolation of the conducted tests to humans.

2.1. Routes of exposure to nanoparticles

Nanoparticles can be introduced into the body either intentionally (*i.e.* as a part of treatment regime or for diagnostic purposes, application of cosmetics and other) or unintentionally – as a result of environmental pollution or accidental release. In the

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latter cases typical entry routes include mainly lungs, skin or gastrointestinal tract (Figure 2). In the case of biomedical procedures usually intravenous (IV), intradermal (ID), intramuscular (IM) and peritoneal injections (IP) are used.
Several studies have been conducted on the toxicology of the nanomaterial inhaled in in the form of aerosol or dry powders [35-37] and applied directly to the respiratory tract of an animal. Results indicated a toxic potential of various nanoparticles introduced via this route. It is a well-established fact that prolonged exposure to some fine particles such as asbestos, coal and silica dust or particulate combustion products leads to detrimental health effects, therefore engineered nanoparticles need special attention in this respect. Specifically, small size and large surface area of engineered NPs may result in a high rate of pulmonary uptake and deposition followed by transportation to systemic sites. Intra-tracheally introduced gold particles into rats were able to cross air-blood barrier and translocate to all organs, variety of tissues, blood and urine, furthermore retained in skeleton indicating deep penetration of the nanoparticles into the body [38].

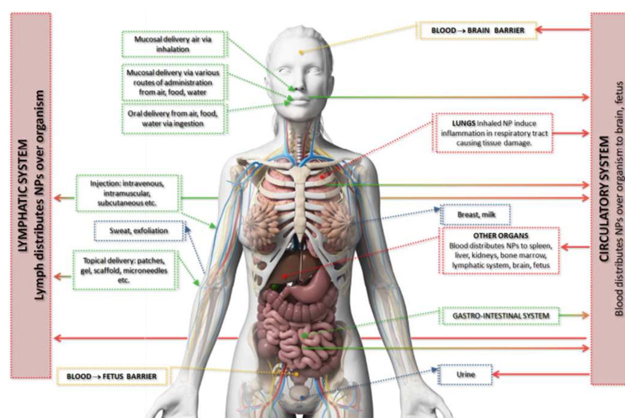

Fig. 2 The NPs may enter human body via various routes (green arrows) and after translocation (red arrows) with the help of blood and lymph, are transported to other organs, eventually crossing blood-brain and blood-fetus barriers. Most of the colloidal NPs are removed from organism through urine and sweat (blue arrows), but some aggregation may occur and prevent successful clearance from the body (see Fig.1).© Sebastian Kaulitzki / fotolia

The mechanisms of NPs translocation through the air-blood barrier has not been explained, but trans-cellular routes have been postulated to play a major role [39]. The clearance on NPs from the lung tissue is maintained primarily by alveolar macrophages [40] and the pulmonary surfactant protein A (SP-A) which binds NPs and enhances the efficiency of NP's phagocytosis, indicating that the fate of the NPs within different body compartments relies on the interactions of the NP's surface with the local biochemical environment [41]. The above-mentioned and other studies, that can be found elsewhere, show that the pulmonary gateway is a very effective entry for nanoparticles that can give access to practically all organs and tissues.
Healthy skin forms an effective barrier that protects from pathogens invasion and action of chemical and physical factors. In general, skin is much less permeable to nanomaterials than lungs. Inorganic nanoparticles commonly used in cosmetics (sun-screens) composed of TiO_2 and ZnO in the size range 50-500 nm are not able to pass through the outer skin layer (stratum corneum) and are considered to be safe for consumers. Similarly, fluorescent labeled fullerene-peptide complex did not penetrate untreated skin. However, with application of some mechanical stress slight penetration could be achieved [42].
In the case of QDs, results differed, depending on the subject animal used in the study. Rat skin was not penetrated by QDs, while such a penetration could be observed for mice and porcine skin as some studies have shown the ability of QD to cross dermal barrier [43-45]. Research on the penetration of gold nanoparticles through the skin have given contradictory results. The study presented by Labouta et al. [46] have shown that AuNPs can pass stratum corneum of human skin via intracellular passages and penetrate deeply into human skin during 24 h treatment [47]. Sonovane et al. [48] showed penetration of 15 nm AuNPs suspended in water through all layers of rat skin. In contrast, Liu et al. observed that human skin was resistant to penetration by 10, 30 and 60 nm AuNPs [49].
Gastrointestinal (GI) route has been studied less extensively so far, probably due to lesser number of potential applications. Nevertheless, GI exposure may also result in NP's uptake and translocation to different organs.
Various types of biological studies require direct and quantitative delivery of various compounds (*e.g.* vaccines, drugs, antibodies, immune sera, cells cohorts, solutions of molecular contrast and labeling agents, nanoparticles, bacteria and viruses *etc*.) into the body. In such cases, injections are a very convenient route for administration. Currently, commonly applicable injection methods in laboratory animals include intradermal (ID), intramuscular (IM), subcutaneous, intraperitoneal and intravenous routes.
Injection at subcutaneous sites results in a formation of the deposit that is gradually cleared with translocation of the injected material to lymph nodes and blood.
Lymph nodes are distributed in large number throughout the body and their major role is filtration of the lymph from waste material and foreign particles. The size of the injected molecules determines the rate of deposit clearance and particles accumulation in lymph nodes. The retention time at the injection site is expected to be longer for larger molecules [50]. Surface chemistry is another factor that influences the transfer of material to lymph nodes, surface moieties enabling efficient interactions with phagocytic cells will promote retention in lymph nodes.
Subcutaneous or intramuscular injections sites are being explored as a route for delivery of vaccines, drugs and for gene therapy in which NPs serve as carriers [51-53]. The intraperitoneal cavity provides an effective route for the introduction of compounds of choice into the body. The IP injection is often considered the application of choice as practically all organs and tissues can be targeted *via* this route similarly to intravenous administration but in contrast to intravenous administration, all injected particles pass initially through lymph nodes that drain the peritoneal cavity before they can reach the blood stream and organs. Intraperitonal route is frequently a method of choice for immunizations of laboratory animals, mediastinaland ipsilateral inguinal lymph nodes were shown to be a locum of primary T cell activation upon injection of protein antigen [54].
Nanoparticles, injected *via* intravenous route are quickly circulated by the bloodstream primarily to the liver and spleen, kidneys, heart, lungs, bone marrow and brain. Retention in blood and organs strongly depends on surface properties of the nanoparticle. Coating, that promote the interactions of NPs with the cell membranes favors the internalization by various types of cells, while the use of biologically inert coatings *i.e.* PEG, results in the prolonged circulation of such NPs in the bloodstream. Particles smaller than 5 nm can be excreted by renal filtration.
Nanoparticles or other foreign particles and compounds are picked up by system of phagocytic cells, called mononuclear phagocyte systems (MPS). MPS is classified as a part of immune system and is composed of several types of phagocytic cells residing in the reticular tissue within the body. The key function of the phagocytic cells is phagocytosis and neutralisation of bacteria, viruses, cells debris, and other undesired species.

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2.2. Mechanisms of cellular and tissue transport of NP's

Nanoparticles can be internalized into the cell *via* processes requiring energy or by passive transport (Figure 3). Process in which cell participate actively in transport of cargo into cell interior is called endocytosis in contrast to the direct diffusion *via* cell membrane. There are several types of endocytosis that differ by mechanism, size and type of cargo to mention a few. The four major types of endocytosis recognized are: (i) phagocytosis, (ii) pinocytosis, (iii) clathrin mediated endocytosis and (iv) caveolae mediated endocytosis [55, 56]. It is worth noting that endocytosis may occur *via* receptor mediated or *via* a non-specific process, pinocytosis, whereby fluid surrounding the cell is taken up regardless of the composition. In receptor mediated endocytosis the molecules are recognized with high specificity before being internalized. Clathrin and caveolae mediated endocytosis has been shown to mediate folate decorated polymeric nanoparticles with a size range 50 nm to 250 nm [57]. Phagocytosis is characterized by internalization of large, particulate materials such as dust particles, and pathogens, This type of endocytosis is performed by specialized cell types and is a key process in innate immunity.

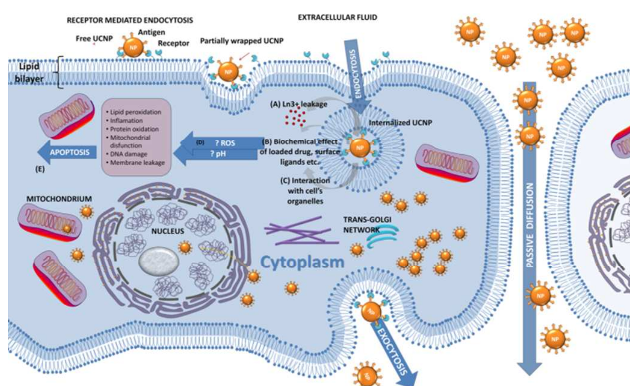

Fig. 3 The biocompatible NPs can be invisible for immune system, and thus can be easily internalized into cells by endocytic pathway ((i) phagocytosis, (ii) pinocytosis, (iii) clathrin mediated endocytosis and (iv) caveolae mediated endocytosis). Potential interactions with cellular organelles are depicted and include (A) Ln^{3+} leakage, (B) interaction of chemical composition of the NPs and ligands with proteins, enzymes and (C) cellular structures, (D) potential ROS generation and chemical environment variations (*e.g.* pH) , which in turn may provoke apoptosis.

Direct translocation of nanoparticles through cell membranes can result in a disruption of the lipid bilayer integrity, which usually results in cellular toxicity. Nanoparticles with cationic surface chemistry are known to possess significant toxicity driven by this mechanism [58]. The necessary condition to employ NPs as a safe vehicle for drug delivery or for intracellular labeling purposes is that penetration through the barrier is not associated with transient membrane leakage.

In addition to factors determining interactions of NPs with cell membranes such as surface chemistry, size and shape, the molecular organization of the coating may play an important role. It has been shown, that nanoparticles with same composition of coating material but different spatial arrangement demonstrated distinct differences in intracellular localization upon translocation through the cellular membrane [59].

While the final conclusions on the nanotoxicity of lanthanide doped nanoparticles will be drawn later, one should be aware that these type of NPs are currently not widely used in technological processes and are not found in the environment. The real potential danger comes from the interest in the use of such UCNPs in biomedical field, owing to their unusual and favourable spectroscopic properties. Since UCNPs are purposely made biocompatible and are stealth for immunologic system, they may easily cross biological barriers. This should be a significant motivation for materials scientists and biologist to unequivocally determine their potential harmful properties, far before they become widely used in novel types of diagnosis and therapy modalities.

2.3. Biological relevance of nanotoxicology

The growing widespread use of biofunctionalized UCNPs, raises some questions about the safety of these highly promising nanomaterials. This is because the very same properties of these nanoparticles that are desirable and potentially useful from a technological or biomedical perspective, are also the properties that may give rise to hazardous, unexpected toxicities. While lanthanide doped UCNPs are basically considered as chemically non-toxic, some concern still exists about the aggregation of the UCNPs within cells and tissues, chemical decomposition of UCNPs, lanthanide ions release or interaction between UCNPs and other compounds *in vivo*.

A direct comparison of nanotoxicity between UCNPs and toxicity from other types of nanoparticles is nearly impossible, due to substantially different physical and chemical properties of UCNPs. Moreover, the rate and mechanism of uptake and clearance are cell/tissue dependent and vary between NPs of different hydrodynamic size, shape, composition, charge and surface functional groups [1, 60]. In addition, for some nanoparticle such as iron oxide NPs, little is known about the NPs metabolism in cellular, endosomal and lysosomal conditions *in vivo*. Recent studies have demonstrated a blue shift and full width at half maximum (FWHM) increase of the fluorescence band of ZnS-shell/CdSe-core quantum dots two years after being administered to rats, indicating the progressing metabolism of these highly toxic nanoparticles [11]. Similarly, based on a comparison between aggregates of UCNPs found in liver and imaged by TEM and quantified by Ion Coupled Plasma (ICP) and the absence of luminescence, Yang et al. [61] proposed that the UCNPs were partially decomposed or damaged inside macrophage cells without significant excretion. Sikora et al. studied internalization of $NaYF_4$:Yb^{3+}, Tm^{3+} and Yb^{3+}, Er^{3+} UCNPs in HeLa cells and concluded that the liposomal transfection factor (Lipofectamine 2000) significantly enhanced the absorption of UCNPs into the cell volume, which was followed by spatial redistribution of UCNPs in the vicinity of the cell nucleus over time [62]. However, the relatively broad size distribution (15-60 nm), the observed aggregation of the UCNPs as well as functionalization by PVP of the UCNPs in an aqueous environment, prevents general conclusions from being drawn and to extrapolate the results to other types of cells and to other possible ligands which are used to functionalize nanoparticles. Studies of the type mentioned above have been few, and interactions of UCNPs at the cellular and subcellular level are far from being understood.

In order to make nanoparticles less- or non-toxic, some methods have been developed to induce bio-incompatibility, which is achieved by coating the NPs with polymers, ligands and detergents to block access to the NPs surface. Changing or adapting the physicochemical characteristics should therefore reduce cellular uptake and bio-availability, limit spreading of free NPs or decrease bio-catalytic effects [63]. In contrast, for some biological applications the biocompatibility of the NPs surface is required. The approach most commonly used to overcome the poor biocompatibility of various NPs (QDs, Ag NP, UCNP *etc.*) has been to deposit a shell of silica, ZnS or a polymer on the NPs. However, it has been shown that silica interacts with cells causing inflammation that may be involved in the initiation of various disorders such as rheumatoid arthritis, sclerosis, lupus or chronic renal disease [64, 65]. Moreover, both SiO_2 or polymer coated NPs tend to aggregate resulting in an increase in their hydrodynamic radius. The increase in size leads to an obstruction of blood flow and capillary vessels blockage [66, 67]. Long-term studies showed that a ZnS shell over CdSe core was ineffective [11]. Although the UCNPs dedicated for biological

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applications are believed to be safe, the potential problem comes from the fact that these UCNPs are designed intentionally to cross biological barriers and circulate within the human body for a prolonged period of time. In this respect, they may become a ‘Trojan horse’ if their potential toxic properties are not recognized. Lanthanide doped UCNPs have not been widely used in commercial assays or tests until now, and there are only a few reports on this subject.

In general, toxicity may be analyzed at consecutive levels of complexity that associates specific phenomena, processes or test methodology [68-71]. These processes, such as exposure and internalization are presented schematically in Fig. 2 and Fig.3. respectively:

a) **cellular level** (cytotoxicity) - apoptosis, necrosis, growth arrest, abnormal morphology, undesired cell signaling or secretory activity. Thorough understanding of these mechanisms and events requires analyses at even more discrete levels: :
   - **molecular level** - misfolding of proteins and disruption of protein conformation leading to inactivation of enzymes, failure of cell signaling or mitochondrial electron transport deregulation, protein aggregation and fibrillation, mutational alterations thought a direct NP-DNA interaction or through reactive oxygen species (ROS) cell stress, NPs coating degradation and ROS induction with following ROS mediated detriments *e.g.* DNA damage, mRNA degradation, gene expression perturbation *etc.*
   - **subcellular level** - membrane disruption or permeability changes, leading to perturbations in intracellular homeostasis, mitochondrial activity perturbations leading to apoptosis.

b) **organ level** - refer to the toxic effects on different organs (mainly kidney, spleen, liver, heart, brain, lungs, skin) that can be assessed or observed upon certain period of exposure. Tests usually include measurement of physiological parameters of a particular organ and examination of its morphology and histological specimens.

c) **whole organism** – assessment of overall body condition, symptoms of abnormal behavior, changes in reproductive potential and other parameters; in some models, changes of body morphology can be indicative *i.e.* zebra fish

d) **environmental** - perhaps the most difficult area of nanotoxicology research due to its complexity, however mass scale production of UCNPs is rather unlikely, therefore their impact on environment is not of prime interest.

These effects originate from either chemical or physical mechanisms. The most dangerous of the chemical mechanisms is a reactive oxygen species generation, which is considered as a primary factor and origin of secondary processes that can ultimately lead to alterations at the subcellular level and subsequent cell damage. Many comprehensive studies and reviews exist on the impact of ROS on living cells [72]. UCNPs have also been successful in research on Photodynamic Therapy (PDT), which relies on ROS generation, allowing for deep tissue treatment and simultaneous fluorescence imaging. Despite the fact, that these UCNPs were impregnated with photosensitizers for that purpose [73-75], similar reactions may take place with tissue chromophores *in vivo*.

Other chemical adverse effects may originate from dissolution and release of potentially toxic ions discussed in section 3.4.

Lanthanide ions are not known to be highly toxic, nevertheless they may interact with proteins and biological molecules. These interactions may disturb the electron/ion cell membrane transport activity causing oxidative damage or lipid peroxidation [68]. In UCNPs, the lanthanide ions are embedded in a dielectric media, therefore surface reactivity should not be as high as in metal or metal oxide NPs. The surface reactivity of the UCNPs should be therefore limited only to lanthanide ions exposed on the surface of UCNPs (Figure 4).

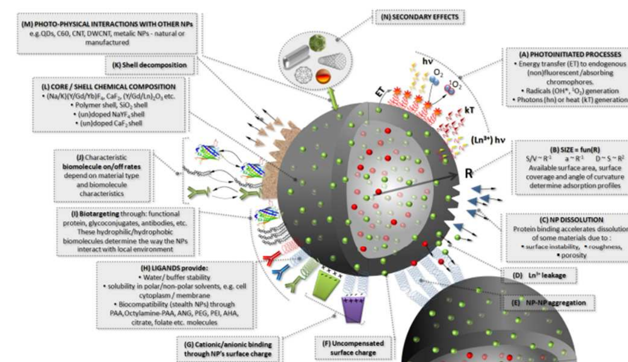

Fig. 4 The expected phenomena occurring specifically on the interface between lanthanide doped nanoparticles and biological environment. (A) Due to energy emission (*i.e.* photons, phonons) and energy transfer between long living Ln^{3+} excited states and long, up to 100Å, Forster distance, the Ln:NPs are capable to ‘FRET’ energy to bound fluorophores (in *e.g.* biosensors) or at worst FRET to photosensitive molecules (*e.g.* porphyrins, capable to produce radicals and singlet oxygen), (B) The small size leads to a large surface to volume (S/V) ratio; large surface curvature and coverage determine adsorption profile of biomolecules, (C) surface instability, roughness and porosity foster surface dissolution of either NPs itself (C) or the protecting shell (K), (D) superficial lanthanide ions may leak from the NPs, (E) Ln:NPs aggregation with other nanoparticles (such as Ln:NPs, Au NPs, CNT, DWCNT etc.), (F) superficial Ln^{3+} ions may be responsible for uncompensated surface charge and electrostatic interactions with environment, (G, H) ligands are responsible for water/buffer colloidal stability and form cationic/anionic binding sites for molecules and biomolecules (I), *e.g.* antibodies are responsible for biotargeting and ‘stealth’ character of the NPs, (J) the protein on-off rate depend on proteins used and surface chemistry, and thus may be susceptible to chemical environment (*e.g.* pH, temperature etc.), (L) the chemical composition of core and shell may be responsible for conventional toxicity, especially upon (K) decomposition, The photophysical interaction (M) between Ln:UCNPs and UCNPs with other natural or engineered NPs (such as QDs, CNT, DWCNT, metallic NPs) may bring (N) secondary effects, resulting from energy transfer UCNPs→QDs, AuNPs, CNTs →e^-, heat) or interaction between these NPs. The hydrophobic or hydrophilic interactions of (bio)functionalized NP’s surface may modify or enhance (E) the aggregation of the NPs. Core (yellow) and shell (blue) hosts can be of different materials (*e.g.* $NaYF_4@CaF_2/SiO_2$ etc.)

Nanotoxicity mechanism at the NP-bio interface may also bring serious adverse effects to the cells and sub-cellular structures. These physical mechanisms include the influence that the NP size or shape may have on membranes integrity, protein conformation or transport processes [68]. In the case of UCNPs with bio-functionalized surface, the bioconjugation allows them to avoid the immune system of the host. This allows the organism to clear the UCNPs; however; it may also be the source of an uncontrolled UCNPs deposition in tissues. A recent study [61] demonstrated that the UCNPs were accumulated and decomposed in the liver, but no indications on prolonged exposure and agglomeration of UCNPs were discussed. Therefore, more studies should be carried out to ascertain the effects of these UCNPs, which are able to cross blood-brain and maternal-fetal barriers.

2.4. National and international regulatory issues towards nanomaterials toxicology

At present a lively debate is taking place in various countries, as to whether nanomaterials and products of nanotechnology require new, specific regulations at the national level. Respective regulatory bodies of the European Union, United States and Australia consider current regulations sufficient for effective toxicological assessment of nanoproducts, although wide range of initiatives are undertaken at national and international level to define and assess the risks and introduce new regulations if deemed necessary.

The European Commission proposed an integrated and responsible approach for Europe to nanotechnology in the Communication "Towards a European Strategy for Nanotechnology" [76] and defined a series of actions for the immediate implementation of a safe, integrated and responsible approach for nanosciences and nanotechnologies [77]. In 2011 the EU Commission announced and adopted the official definition of nanomaterials [78], followed by the Communication on the Second Regulatory Review on Nanomaterials adopted in 2012 [79] and published a paper entitled "Staff Working Paper on nanomaterial types and uses" [80]. However, a fundamental European Union Regulation in that matter is Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) adopted in 2006 [81]. REACH provides an over-arching legislation applicable to the manufacturing, requirements for market distribution and use of the substances on their own, in preparations of other derivative substances or in final products. Also the Scientific Committee for Emerging and Newly Identified Health Risks (SCENIHR) stated that although the existing toxicological and ecotoxicological methods are appropriate to assess many of the hazards associated with the products and processes involving nanoparticles, they may not be sufficient to address all of the hazards [82, 83].

In the United States, the National Nanotechnology Initiative (NNI) was officially created to widen applications of nanotechnology [84]. The NNI brings together the expertise of all participating agencies creating a framework for shared goals, priorities, and strategies. The United States Food and Drug Administration (FDA) stated in 2007 [85], that the FDA does not establish an official regulatory definition of nanotechnology. However, already in 2011, in response to growing number of products on the market, the FDA issued its preliminary guidelines for assessment whether a product, which is a subject to FDA regulation, contains nanomaterials or involves the application of nanotechnology [86]. In 2012, FDA issued for public comment two product-specific draft guidance documents to address the use of nanotechnology by the food and cosmetic industries [87, 88]. Finally, in its 2012 Statement [89] the FDA stated, that although nanomaterials can have different properties than their conventionally-scaled counterpart materials, the FDA - in general - still considers the current framework for safety assessments appropriate for a variety of materials, including nanomaterials.

In Australia, the National Nanotechnology Strategy (NNS) [90] aims to establish an environment, that allows Australia to capture the benefits of nanotechnology while addressing the issues impacting on successful and responsible development of nanotechnology. In 2010, Australian Government officially released the National Enabling Technologies Strategy (NETS) [91] which replaced NNS, to provide a framework for the development of nanotechnology. The Therapeutic Goods Administration (TGA) states, that, to date, the existing regulatory framework of the TGA has proved more than adequate to identify, assess and manage the risks associated with therapeutic products that incorporate nanotechnologies. However, the TGA recognizes the challenges of the nanotechnologies, therefore continued development of high quality scientific expertise will be indispensable.

In 2007 the Government of Canada, through Environment Canada issued a publication "New Substances Program Advisory Note 2007-06" [92], to inform all stakeholders of the requirements for the substances under the New Substances Notification Regulations (NSNR). The regulations apply to new nanomaterials just as any other substance. In the same time Canada with international partners through the International Organization for Standardization (ISO) is developing standard terminology and a formal nomenclature system for nanomaterials. Environment Canada and Health Canada have developed new legislatory proposals and a regulatory framework for nanomaterials with a working definition of nanomaterials [93, 94], however at present, there is no definitive system of nomenclature for that type of products. Until a nomenclature system is established, The Government of Canada has issued an advisory note describing new and existing nanomaterials under the current regulations [95]. Also The New Substances (NS) program of Environment Canada is responsible for conducting risk assessments and administering appropriate risk management, when necessary, of chemical substances under the authority of the New Substances Notification Regulations (NSNR).

3. Lanthanide doped nanoparticle bioprobes

Like many other inorganic NPs, upconverting nanoparticles are not easily prone to biodegradation. At this time there is a growing concern about the possible health issues related to nanomaterials. Therefore, as UCNPs mature and are integrated in the biomedical field an understanding and assessment of their long-term impact on biological systems such as cells and multi-cellular organisms is paramount. As has been the case in drug development some of the properties which are desirable and potentially useful such as the ability to cross biological barriers or the high degree of surface reactivity may also be a source of unexpected and hazardous toxicities [96]. Similarly to other nanoparticles, chemical composition, shape and size, high chemical reactivity, and large surface-to-volume ratio of UCNPs may be determinants of potential toxicity [1]**.** In the present work we survey the available results concerning studies on UCNPs toxicity *in vivo* or *in vitro* applications. Although a precise assessment of the different UCNPs' impact on living organism and cell cultures is difficult, the following sections provide a summary of the results that have been obtained with respect to the toxicity and biodistribution of UCNPs (section Nanotoxicity studies on Ln:NPs and Ln:UCNPs) and lanthanide ions (section Lanthanide ions toxicity) is provided for scholarly purposes.

3.1. Upconversion

Conventionally, lanthanide emission is observed upon direct excitation into an excited state, followed by emission and return of the excited ion to its ground state producing light of lower energy (Stokes emission). An alternative method to observe lanthanide emission is *via* the process of upconversion. The 'upconversion' stands for generation of higher energy photons (anti Stokes) such as UV, visible or near infrared (NIR) using a low energy photons such as NIR. It is a multiphoton process involving at least two excitation photons, where the absorption of these photons is sequential and not simultaneous. There are three major mechanisms for upconversion, namely most efficient energy transfer upconversion (fr. *Addition de Photons par Addition de Photons par Transferts d'Energie)* and the less efficient excited state absorption and photon avalanche [97]. The use of NIR light to generate emission in the UV, visible and NIR using NIR excitation has provided numerous opportunities in the biomedical field [98-117] with potential applications in bioimaging, cell and tissue labeling, biodetection, therapy and multiplex analysis in which UCNPs may substitute traditional dyes. UCNPs offer a number of advantages for application in the biological

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science: (i) enable the detection of the signal in an autofluorescence-free environment [118] leading to higher signal to noise ratio (SNR) and provides a deeper yet noninvasive penetration of the radiation into biomatrices (Figure 5) [119], (ii) narrow emission bands, (iii) resistance to photobleaching, (iv) absence of blinking, (v) large Stokes shift, (vi) long (micro-to milliseconds) luminescence lifetimes, (vii) as well as high chemical and physical stability [108].

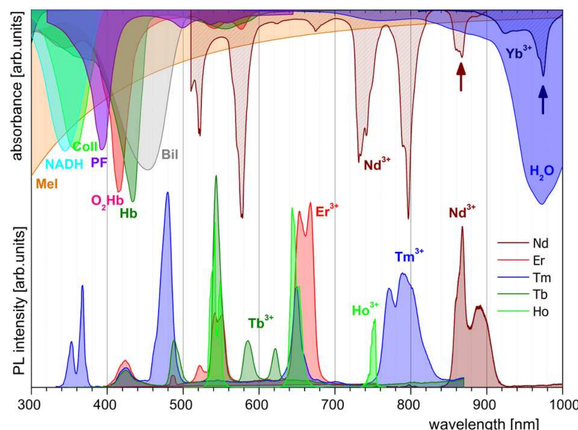

Fig. 5 Spectral overlap between absorption bands of endogenous/exogenous chromophores (top axis, Mel - melanosomes, NADH - Nicotinamide adenine dinucleotide, Coll - collagen, PF - Photofrin©, O_2Hb - oxygenated hemoglobin, Hb - hemoglobin, Bil - billirubin) as compared to absorption spectra of Yb^{3+} and Nd^{3+} (top axis) and Nd^{3+}, Tm^{3+}, Er^{3+}, Ho^{3+} emission (bottom axis). Arrows indicate most typical excitation of UCNPs in $Yb^{3+}\rightarrow Tm^{3+}$/ Er^{3+}/ Tb^{3+} *etc.* (at 980nm) and $Nd^{3+}\rightarrow Yb^{3+}\rightarrow Tm^{3+}$/ Er^{3+}/ Tb^{3+} *etc.* (at 796nm) configurations. The Tm^{3+} emission at ~350 nm (particularly strong in $LiYF_4$) may overlap with NADH nucleotide or the Soret band of PS (porphyrin based PhotoSensitizers such as PpIX). The spectra are not in scale and are provided to demonstrate general overlap between respective spectra.

3.2. Size and structural properties

It is widely accepted, that many nanomaterials show size- and structure-dependent toxicity. For example small – 1.4 nm – gold nanoparticles have been shown to be highly toxic and inducing rapid cell death, whereas larger 15 nm AuNPs have been found safe [120]. Similarly, owing to oxidative stress, the alveolar macrophages cytotoxicity from 15 nm particles was much higher than for larger silver NPs [121]. Smaller NPs typically demonstrate prolonged circulation and retention time, increased translocation and greater epithelial effects. Crystal structure also affects toxicity for example the two phases of TiO_2 NPs exhibited different toxicity, namely anatase TiO_2 NPs induced necrosis regardless of size, while rutile TiO_2 NPs initiated apoptosis through the formation of ROS [122]. For more comprehensive reviews on the cytotoxicity of gold, silver, CNT, MWCNT the readers are referred to ref. [27].

Sodium yttrium fluoride and sodium gadolinium fluoride ($NaLn1_xLn2_{1-x}F_4$, where Ln1=Y^{3+}, Gd^{3+}, and Ln2 (dopant) = Yb^{3+}, Tm^{3+}, Er^{3+}, Ho^{3+}, Tb^{3+} *etc.*) upconverting nanoparticles are among the most efficient upconverting fluorides hosts and have been extensively studied and reviewed in recent years because of their potential application in biological systems [98-117]. In contrast to conventional luminescent probes, lanthanide doped UCNPs offer a number of advantages (see previous section). Long emission lifetimes (micro to milliseconds) allows time-gated detection, thereby short-lived autofluorescence emitted by biological specimens on excitation can be completely separated from the long luminescence lifetimes of the UCNPs eliminating autofluorescence background

Both $NaYF_4$ and $NaGdF_4$ are known to crystallize in either the cubic (Fm3m, a = 5.470 Å) or the hexagonal (P63/mmc a = 6.035(± 0.002) Å, c = 3.614(± 0.001) Å) phase with the latter being more thermodynamically stable. Cubic or hexagonal phase may be obtained by changing the reaction parameters (temperature, reaction time). In the case of the most commonly used synthetic route *i.e.* thermal decomposition, the rate of addition of the precursors also plays a role. The type of capping ligand has also been shown to influence the physical properties (Figure 6). The solvent has been shown to modulate the morphology of the UCNPs [123]. Hexagonal $NaYF_4$ nanoparticles, have been synthesized showing different morphologies, such as rice-like, needles, corn-flakes, hexagonal *etc.*, typically spherical, cubic or diamond like structures have been exploited in biomedical applications. Most often, UCNPs in the size range 5-50 nm have been synthesized however, the hydrodynamic size depends on the ligands used and the formation of the protein corona on the nanoparticles thus the size may increase substantially (up to 50 nm or more). Very often, core-shell UCNPs (such as $NaYF_4$@ $NaYF_4$/ CaF_2/ SiO_2) have been synthesized in order to improve upconversion efficiency [124-126] or to tune the upconversion properties [127-129]. Placing a shell/shells on the core UCNPs increases the size, but our studies have shown the shell thickness may be easily controlled to approximately 2 nm, which is suitable to passivate the core and enhance the upconversion efficiency [126].

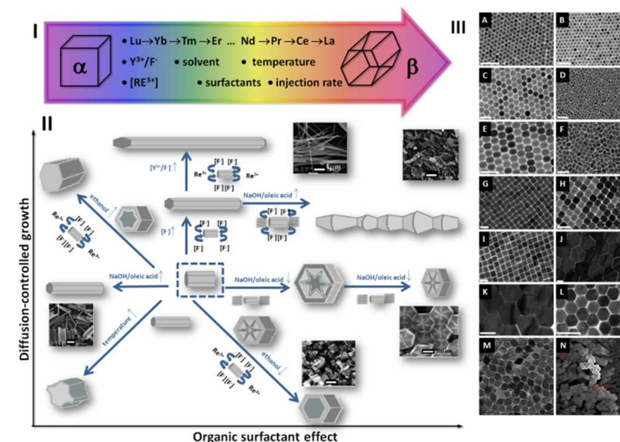

Fig. 6 (a,b) General structural properties and morphology of Ln:NPs with the dependence of $NaYF_4$ NPs structure on synthesis conditions, (b) possible morphologies of different $NaYF_4$ UCNPs. (pictures (II) reprinted with permission from Macmillan Publisher Ltd: [Nature], Ref. [130], copyright 2009; pictures (III A-L) reprinted with permission from PNAS, Ref.[131], copyright 2010; pictures (II) reprinted with permission from John Wiley & Sons [Chemistry - A European Journal], Ref.[132], copyright 2009; pictures (III M) reprinted with permission from J. Capobianco, unpublished)

3.3. Surface chemistry properties

Upconverting nanoparticles show various chemical, physical and optical properties depending on the capping ligand. The ligands are usually responsible for stabilization of UCNPs (and nanoparticles in general) in solutions and prevent the aggregation. Another very important role of the functional group on the UCNPs is to provide the nanoparticle's surface to be hydrophilic or amenable to modification for a facile dispersion in biologically relevant media. Functional groups at the surface of UCNPs such as thiol, amine and carboxylic acid permit a functionalization with receptor molecules, that would allow them to be integrated into the imaging and biomedical fields [133-140]. In

addition, the functional group on the surface of the UCNPs may enhance their ability to penetrate cell membranes, and facilitate their circulation in the body.

3.4. Rare earth ions toxicity

The lanthanides are regarded as relatively non-toxic elements. For example the LD_{50} of lanthanide chlorides ($LnCl_3$) or nitrates ($Ln(NO_3)_3$) following intravenous, intraperitoneal or per os (PO) administration, is higher than 10 (IV), 450 (IP) and 1100 (PO) mg per kg body weight [141]. The clearance of the lanthanides from an organism after intravenous administration follows a three-phase model; fast, intermediate and slow half-times equal respectively from hours (single digit) to a day (fast), hundreds of hours to days (single digit) (intermediate) and hundreds to 850 days (slow phase) for lanthanide citrates. Notwithstanding the potentially encouraging properties – *i.e.* high LD_{50} values, lanthanide compounds have shown to cause a number of health related problems. Chronic exposure to rare earths (lanthanides) dust may lead to pneumonitis and acute inflammation. Intravenous administration of Ln chlorides may increase vascular permeability for low molecular weight substances and cause necrosis in the liver and spleen [142]. A number of hepatic and liver associated biochemical changes were observed following intravenous administration of lanthanide chlorides. These changes were dependent on the lanthanide chlorides being injected and contradictory behavior was noticed for various lanthanide chlorides [143]. For example RNA polymerase II activity decreased under exposure to Pr^{3+}, Nd^{3+}, Sm^{3+}, and increased for Gd^{3+}, Dy^{3+} and Er^{3+} nitrates. Severe effects of lanthanide chlorides has been found on blood content (decreasing the content of cholesterol, collagen, elastin *etc.* reducing the atherosclerotic progress), and the decreased activity of some enzymes (*e.g.* Ca^{2+}/Mg^{2+}-ATPase, NADH dehydrogenase) [144, 145]. No serious effects were found in spermatogenesis in rats [146] however animals injected with lanthanide citrates during lactating periods or pregnancy, demonstrated significantly reduced body weight of progeny complete stoppage of excretion or decrease in an average litter size, but no malformations were observed in fetuses [147, 148]. There has been no report of carcinogenicity or mutagenicity due to the lanthanide ions [141]. In lanthanide doped UCNP, lanthanide ions are embedded into a dielectric matrix, and basically cannot freely diffuse into cells, unless the UCNPs undergo decomposition *in situ*. Results of recent study on toxicity of rare earth oxides allowed to conclude that dissolution of REO nanoparticles in lysosomal compartment released RE (rare earth) ions that upon precipitation with cellular phosphates formed needle like structures. Interestingly, toxic effects were shown to arise from dephosphorylation of membrane phospholipids rather than mechanical stress caused by formed crystals. Prior coating of nanoparticles with phosphates effectively prevented toxicity [149].

4. Nanotoxicity studies on Ln:NPs and Ln:UCNPs

To date the majority of the data on UCNPs toxicity has been obtained from *in vitro* studies on cell cultures [10, 150-155], and lesser number of *in vivo* toxicity studies have been reported [10, 61, 151, 152, 156-161]. Data on the effects of lanthanide doped nanoparticles at the cellular or sub-cellular levels is also very scarce [62].
Recent studies investigate the properties of nanoparticles of different chemical composition, size, morphology, structure, concentration of lanthanide ions and surface ligands. Therefore, the comparison of the results from the available literature for an assessment of the potential toxicity of UCNPs is not an easy task. In addition, there are many questions, which need to be answered with respect to the interaction of UCNPs in biological systems. Various cell culture have been used for *in vitro* cytotoxicity studies. These studies do not reflect long-term safety and cytotoxicity, however they do suggest a need for *in vivo* animal studies [1, 100, 162]. Recently, a new field of toxicology – nanotoxicogenomics [163] - that uses cDNA microarray technologies to study the impact of nanoparticles on global gene expression profiles of cells and tissues [1, 163], may provide new and important insight into the toxicity of UCNPs in the future.
Moreover, there is a paucity of studies on how UCNPs interact with other NPs (both natural and man-made). This is because studying the potential risk of the synergistic interaction between one or more types of nanoparticles in combination with other co-pollutants or naturally occurring colloids and proteins / enzymes present in living cells is extremely difficult. Nanoparticles may not initially exhibit the toxicity to living species, but in their lifecycle they could become toxic. The currently available results of *in vitro* and *in vivo* nano-toxicity studies are discussed in the following sections and summarized in Table 1 and Table 2, respectively.

4.1. *In vivo* studies

One of the most comprehensive long-term studies of toxicity of UCNPs *in vivo* has been reported by Xiong et al. [156]. The authors investigated the biodistribution and toxicity of $NaYF_4$:Yb^{3+}, Tm^{3+} coated with PAA in mice. Early after intravenous injection UNCP were cleared from circulation and could be detected in the liver, spleen and to lesser degree in lungs, trace signal was observed in kidney and heart. During the first 24 hours accumulation of UCNP occurred initially in the spleen and slowly decayed in the liver. After two weeks, the emission signal from UCNPs was still detectable in the liver and spleen, indicating the crucial role played by these organs in the clearance of UCNPs from the body. The emission signal from the UCNPs was still detectable from the intestinal tract three months post injection. The authors stated that after four months nearly no luminescence signal from the UCNPs was detected (Figure 7) and that the mice showed no apparent symptoms of health deterioration. Analysis of blood smears indicated that the count and shape of the red blood cells, platelet, and white blood cells was normal. Also diagnostic markers of the liver function (alanine aminotransferase - ALT, aspartate aminotransferase - AST and total bilirubin) and kidney (creatinine and urea) and body weight were not different from mice in a control group. Silmilarly , no changes were observed in food intake, water consumption, fur colour, exploratory behavior, activity and neurological status. Only minimal hyperplasia in the periarteriolar lymphoids sheath (PALS) of spleen white pulp as an effect of nanotoxicity was noticed.

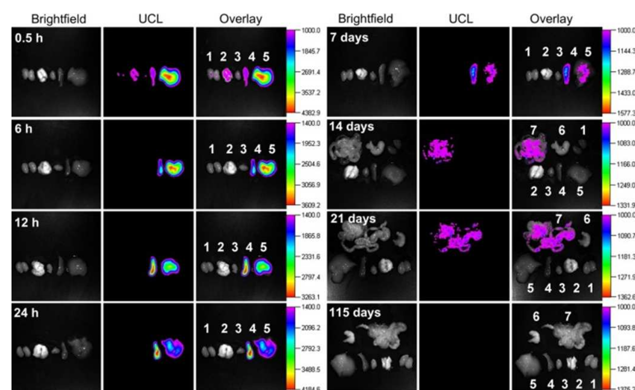

Fig. 7 Real-time *ex vivo* up-conversion luminescence (UCL) imaging. The uptake and biodistribution of PAA-UCNPs (15 mg kg^{-1}) after intravenous injection at different time points shows early accumulation in liver and spleen. At 115 days post-injection, nearly no UCL signal was observed in the mice, showing that most of the PAA-UCNPs were excreted from the body of the mice. Biodistribution of PAA-UCNPs 1: kidney; 2: lung; 3: heart; 4: spleen; 5: liver; 6: stomach; 7: intestines.

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Yang et al. [61], conducted a comprehensive *in vivo* study on PAA and PEG coated β-$NaYF_4$:Tm^{3+}, Yb^{3+} UCNPs. The authors showed that no organ damage or lesion in mice intravenously injected with a 20 mg kg^{-1} dose, based on histology of examined organs. In addition, serum biochemistry, hematology, hepatic enzymes levels indicated no atypical parameters. Interestingly, aggregates of nanoparticles were found in the liver (7 days post injection, imaged by TEM and quantified by Ion Coupled Plasma (ICP) method) but with no apparent luminescence. It was suggested that UCNPs could be partially decomposed or damaged inside macrophage cells that resulted in the loss of their luminescent properties. This conclusion needs further investigation, since similar observation was not reported by other researchers, instead previous reports suggested fast and effective clearance of nanoparticles from the body. Therefore a question arises whether observed decay of luminescence may be attributed to effective excretion of nanoparticles from a body or may be a result of a partial decomposition while deposits may persist in a body for a long time. Both types of nanoparticles (UCNPs-PAA and UCNPs-PEG) showed similar uptake by the liver, however accumulation in the spleen and lung was lower for the UCNPs-PEG . The pharmacokinetics of UCNPs in blood was shown to be dependent on coating material. The clearance half-time of UCNPs-PEG and UCNPs-PAA from the blood showed a two phase decay equal to 5.1/13.1 and 0.13/3.5 minutes respectively. It is worth mentioning that half-time clearance from the circulation is an important parameter determining the usefulness of functionalized UCNPs for certain applications, i.e. imaging of small metastatic sites throughout the organism. The small-8 nm $NaYF_4$:Er^{3+}, Yb^{3+} PEG coated nanoparticles labeled with ^{153}Sm were investigated by Cao et al. [17] to ascertain the biodistribution and blood retention time in Kunming mice. Following intravenous injection of PEG-UCNPs the biodistribution was assessed for different organs at 1 h, 6 h, 12 h, 24 h and 48 h post injection. As expected, the majority of UCNPs-PEG was detected in the liver and spleen. Significant amount of UCNPs-PEG was detected in the kidney (12.48 ±1.80% IDg^{-1}) and urine (5.28 ±0.2% IDg^{-1}). A much lower amount was detected in the small intestine (0.06 ±0.02% IDg^{-1}) and the large intestine (with feces, 0.21 ±0.04% IDg^{-1}), which indicates a faster renal excretion.

Chatterjee al. [151] studied the impact of PEI-coated UCNPs on rats. UCNPs injected into animals accumulated quickly in lungs. The amount of UCNPs was reduced after 24 hours in lungs while the concentration in spleen increased. After 7 days the UCNPs were undetectable in animals. Biodistribution of UCNPs conjugated with FA was tested by Xiong et al. [135]. The UCNPs accumulated mostly in cancer cells because FA guided the UCNPs to FA receptors on cancer cells surface. However, small amounts were found in the liver and spleen, while in the kidneys and the heart they were rarely detected. Xing et al. [13] showed that $NaYbF_4$ UCNPs administered to mice with at 150 mg kg^{-1} (Yb^{3+}) dose were excreted with feces and urine up to 7 days after injection. After one month, no fluorescence from $NaYbF_4$ was observed showing that all UCNPs were removed from the body. The biodistribution studies showed that $NaYbF_4$ accumulated in liver and spleen (the highest amount of UCNPs after 0.5 h and 24 h after administration), smallest amount of UCNPs was detected in lungs and kidney (after 24 h), but after 30 days no UCNPs were present in these organs. Histology analysis proved that no damages or toxic effects to organs were caused by long distribution time of UCNPs *in vivo*.

In another study, two hours after injection, the amount of UCNPs in the liver was decreasing, while raising in the spleen. Small amount of UCNPs were detected in the heart, lungs, kidney and muscles [164].

Orally administerd (400 µL of 20 mg mL^{-1} UCNPs) PEGylated Yb_2O_3:Er^{3+} upconverting nanoparticles were studied *in* Kunming mice and were considered safe. The mice showed no histological changes and tissue damage in organs after 30 days. The bio-distribution analysis revealed rapid elimination of these UCNPs 2 days after oral administration *via* renal and fecal excretions.

Toxicological studies of $NaLuF_4$:$^{153}Sm^{3+}$, Yb^{3+}, Tm^{3+} coated with 6-AHA revealed no signs of toxicity [165]. Intradermal injection of 20 µl UCNPs in 0.2 mg mL^{-1} PBS had no adverse effect on mice. Major organs (heart, liver, spleen, lung and kidney from mice exposed to UCNPs were not affected 7 days after injection when compared with organs from the control group. Levels of a major hepatic indicators; alanine aminotransferase (ALT), aspartateaminotransferase (AST), glutamyltranspeptidase (GGT), globulin (GLO), total bilirubin (TBIL) and total bile acid (TBA) were similar to those in untreated mice.

Gadolinium is the most paramagnetic ion in the periodic table with seven unpaired electrons offering strong interaction with the proton spin. Therefore, gadolinium based chelates in which gadolinium is present as the Gd^{3+} ion, are among the best and most used contrast agents (CAs) for the magnetic resonance imaging (MRI) (others include Mn^{2+} and Cu^{2+}). The Gd^{3+} ion possesses nine coordination sites however in the complex, which it is normally used in MRI, it is chelated to eight atoms with the last coordination site reserved for interactions with water protons. Recently the concept of multimodality of nanoparticles has been proposed, which refers to the ability of nanoparticles to carry out multiple functions for example optical and magnetic resonance imaging.

An area of concern for Gd^{3+} based MRI contrast agents is the potential toxicity associated with leaching of free Gd^{3+} ions from the complex. Recent studies have shown nephrotoxicity of gadolinium-based contrast agents [166, 167]. With this in mind, $NaGdF_4$ upconverting nanoparticles have been developed as an alternative to 'bare' gadolinium-contrast agents. These inorganic nanoparticles offer several advantages. The Gd^{3+} ions are tightly bonded in an inorganic crystal thus the likelihood of leaching is minimized and at the same time they can serve as bi-functional optical/T_1-MR imaging probes.

The issue of toxicity of Gd^{3+} ions has been addressed not only for UCNPs but also for a variety of gadolinium-based nanoparticles. Recently, Yang et al. [10] reported on the application of ^{153}Sm-doped $Gd(OH)_3$ nanorods as a potential MRI contrast agent. In the publication the authors also report on the toxicological studies, which were carried out. *In vitro* test on KB cells demonstrated no harmful effect of the nanorods on cellular proliferation and viability. The results on the uptake and retention of $Gd(OH)_3$ nanorods in Kunming mice showed that the liver, spleen and lung were the primary targets and the retention time was very short. Long-term studies showed, that injection of up to 100 mg $kg^{-1}$of $Gd(OH)_3$ nanorods had no adverse effect on the mice up to 150 days after exposure. For example, no changes in body weight, and no abnormalities in the histological or hematological parameters were observed.

Studies on mice showed the accumulation of Gd^{3+} ions from Gd_2O_3:Er^{3+}, Yb^{3+} nanoparticles in the mononuclear phagocyte system (MPS) of the liver and spleen 24 h post-injection however the mice cleared the UCNPs within 30 days. Histological examination did not show any tissue damage, and hematology and biochemical blood assay did not demonstrate any changes between mice exposed to uncoated and coated Gd_2O_3:Er^{3+}, Yb^{3+} nanorods.

It is also important to assess the risk of UCNPs to the ecosystem and their effect on the food chain. The uptake of UCNPs from aqueous colloidal solution by plants was first investigated by Haase et al. [168]. The authors watered *Phalaenopsis* and *Arabidopsis* plants using a solution of $NaYF_4$:Yb^{3+}, Er^{3+} UCNPs to study their uptake by the roots. The effect of UCNPs on plants was also studied by Li et al. [169] using mung bean as a model. They demonstrated that low concentration of ^{153}Sm-labeled citric

acid-coated UCNPs (<10 μg mL^{-1}) increases the bean sprout growth, which is possibly due to changes induced by the UCNPs in the uptake of water by the plant. However an increase in the concentration of UCNPs (100 μg mL^{-1}) resulted is an inhibition in growth. The majority of the UCNPs accumulated in roots and seeds. Plants treated with UCNPs were used for feeding mice and the animals showed no evidence (observational, histological, hematological or biochemical) of toxic effects after 7 days.

Caenorhabditis elegans have been used to evaluate the *in vivo* toxicity of $NaYF_4:Ln^{3+}$ [157, 158, 161]. The survival rate of *C. elegans* was investigated at three different concentration of $NaYF_4:Yb^{3+}$, Er^{3+}@PEI (1, 2.5 and 5 mg mL^{-1}) and at two different times, 3 or 24 hours, after ingestion. The result showed no significant harmful effect on *C. elegans* treated with a low concentration (1 mg mL^{-1}) of UCNPs and a short time period (3 hours). Also there was no difference in warms behavior after 20 hrs treatment with high concentration of UCNPs (5 mg mL^{-1}) when compared with untreated warms. The authors postulate that at higher concentrations toxicity may result due to the positively charge PEI which is known to disrupt the cell membrane. It has also been shown that difference in the size or shape or surface ligands of the UCNPs has no toxicological effect on *C. elegans*. Studies on the next generation of the worms did not show any fluorescence under 980 nm excitation and the UCNPs did not affect the oogenesis or embryonic development [158]. Zhou et al. [161] assessed the effect of UCNPs on *C. elegans* by evaluating their toxicity on protein expression, life span, egg production, egg viability and growth rate of the worms. The authors found no significant difference between *C. elegans* incubated with UCNPs and those that were not.

The up-converting nanoparticles, $LaF_3:Yb^{3+}$, $Er^{3+}@SiO_2$,were tested on Zebrafish embryos to assess their toxicity [159]. The UCNPs were injected through the chorion into the yolk sac. The results differed from those usually obtained from the experiments on either cell culture or mice. No toxicological effects were observed post injection of up to 100 μg mL^{-1} of the UCNPs. However, at a dose of 200 μg mL^{-1} of UCNPs, malformations and other developmental abnormalities such as shortened larval body length were observed. The authors also reported delayed embryonic and larval development, which may be related to the binding of La^{3+} ions to the DNA inducing DNA damage. Expression of *sepn1* gene in embryos injected with $LaF_3:Yb^{3+}$, $Er^{3+}@SiO_2$ was reduced in comparison to the control group. This led the authors to postulate that La^{3+} ions may enter the nucleus and alter the gene expression. Furthermore, the hatching time and hatching success rate decreased with increasing concentrations of UCNPs. The UCNPs were found in the intestine, but not inside the cells 24 hours after injection, which indicates that the UCNPs were not able to penetrate the intestinal cell membrane (neither by active transport nor by phagocytosis) and can be excreted. Another very important result is that the UCNPs were resistant to action of digestive enzymes, thus the release of free lanthanide ions is unlikely.

Jang also studied the toxicity of $NaYF_4:Ce^{3+},Tb^{3+}$ on Zebrafish embryos [160]. The embryos were incubated with 0.5 pM – 5 nM nanoparticles suspension 24, 48 and 72 hours post-fertilization, The phenotypic abnormalities, localized cell death, and developmental abnormalities of the heart were assessed. The group exposed to less than 50 pM nanoparticles showed no significant abnormalities at 80 hours post-fertilization. A dose over 500 pM caused bent tail phenotype. A decrease in blood circulation was observed in 12.5% of Zebrafish embryos treated with a concentration of 0.5 and 1 nM nanoparticles 24 hours post-fertilization and growth retardation was observed in 10.9% and 28.6% of embryos treated with concentrations of 500 pM and 750 pM of nanoparticles respectively. The effect of the nanoparticles was examined at an individual organ level using cardiac myosin light chain 2(cmlc2) transgenic Zebrafish. A dose of less than 500 pM of nanoparticles caused no changes in morphology of the heart in comparison to the control group. Moreover, lanthanide doped nanoparticles showed 10 times lower toxicity than QDs (Nanodot HE-series 100-620 nm).

The cellular uptake of silica coated UCNPs was also studied by Zhang and Jalil [170]. They carried out the cytotoxicity test by assessing the mitochondrial function and membrane leakage. The *in vivo* biodistribution studies showed that the lung and the heart accumulated the majority of the UCNPs but the concentration of UCNPs in these organs decreased during the first 24 hours. 7 days after injection, UCNPs were almost completely excreted from rats' bodies.

These studies have been summarized in Table 1, where biodistribution experiments with luminescent Ln^{3+}:NP and UCNPs are presented. The nanoparticles' size, shape, functional molecules on the NPs' surface, injection conditions, animal/tissue type and tissue deposition are provided together with short comments.

Although the presented results appear to be comprehensive and reliable in assessing the toxicity of Ln:NPs using standard protocols, the special properties of NPs, especially UCNPs, where their bioactivity is highly influenced by the variety of external factors requires much more complex studies of their potential toxic activities and should raise questions on the validity of assumptions being made up to date.

While the *in-vivo* toxicity studies of Ln:NPs are relatively coherent in stating about the non-toxic character of these nanomaterials, there is a continuous debate on how to quantify amount of NPs (whether by surface, mass or number of NPs), or how to account the different surface chemistry (which is responsible for corona formation, bio-interactions, triggering and bio-signalling, aggregation, stealth function etc.). Little is known about passing blood-brain or blood-fetus barrier by the UCNPs and following impact of such NPs in living organisms.

Moreover, at the era of widespread use of nanomaterials, humans may be also exposed to different types of man-made synthetic nanomaterials, which mutual interaction with bio-chemical environment and under exposure to physical field (such as magnetic or electromagnetic fields) may be extremely difficult to predict and simulate in sterile laboratory conditions. These interactions in turn may bring some changes to the NP structure, such as ligand removal or UCNPs aggregation, which may in consequence lead to secondary toxicity or tissue malfunctioning, even though the UCNPs are basically considered to be non-toxic.

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Table 1. Summary of *in vivo* biodistribution experiments with luminescent Ln^{3+}:NP and UCNPs. ID g^{-1} – Injected dose per gram of tissue; mg g^{-1} L^{-1} – concentration of ion (mg L^{-1} measured by ICP) injected per gram of body weight.

NPs	Biofunctionalization/ Bioconjugation	Injection conditions	Animal / tissue	Tissue deposition [%]	Comments	Ref.
$NaYF_4$:25% Yb^{3+}, 0.3% Tm^{3+} ϕ=21±0.5 nm	SiO_2 L= 8±1.5 nm thick	Intravenous injection 10 mg kg^{-1} BW	Female Wistar rats (200-250g) Heart: Lung: Spleen: Kidney: Liver: Blood:	ICP of Y^{3+} [mg g^{-1} L^{-1}] at 10 min; 30 min; 24 h and 7 days post injection 10 min; 30 min; 24h; 7 d 18; 10; 1; 0 29; 19; 2; 0 2; 7; 3; 0 7; 4; 5; 0 2; 1; 0; 0 2.5; 1; 0; 0	health status and behaviour of all the animals was normal throughout the study	170
$NaYF_4$:20%Yb^{3+}, 1%Tm^{3+} ϕ~11.5 nm	poly(acrylic acid) (PAA)	Intravenous injection 15 mg kg^{-1}	athymic nude mice Heart : Lung : Spleen : Kidney : Liver :	ICP of Y^{3+} [% ID g^{-1}] at 0.5 h, 24 h, 7 days, 115 days post injection 0.5 h; 24 h; 7 d; 115 d 0.02; 0.05, 0.01, 0.01 0.15; 9.1; 0.08; 0.02 1; 7; 3.5; 0.03 0.02; 0.03; 0.01; 0.01 0.8; 0.5; 0.4; 0.02	no toxic effect on the cells/ mice was found, slight hyperplasia in the periarteriolar lymphoid sheath (PALS) of white pulp	156
$NaYF_4$:Yb^{3+}, Er^{3+} ϕ = 50 nm	poly(ethyleneimine) (PEI)	Intravenous injection 10 mg mL^{-1}	Female Wistar rats (200–250g) Heart : Lung : Spleen : Kidney : Liver : Blood :	deposition – ICP of Y^{3+} [% ID g^{-1}] at 0.5, 24 h, and 7 days post injection 0.5 h; 24 h; 7 d 7; 2; <1 19; 1; <1 4; 2; <1 2.5; 1; <1 1; 1; <1 <1; <1; <1	no toxic effect on the cells/ mice within reasonable concentrations	151
$NaGdF_4$: Tm^{3+}, Er^{3+}, Yb^{3+} ϕ=30-40 nm	oleic acid oxidized to azelaic acid ϕ up to 60 nm	tail vein injection 150 µL, 1.0 mg mL^{-1} per animal	mice Heart : Lung : Spleen : Kidney : Liver : Muscle :	deposition – ICP of Gd^{3+} [% ID g^{-1}] 40 min post injection 0.63 1.21 12.4 0.57 7.51 0.79	high relaxivity of 5.60 s^{-1} mM^{-1} and were successfully applied as contrast agents for magnetic resonance imaging (MRI) *in vivo*	171
β-$NaYF_4$: 20%Yb^{3+}, 2%Tm^{3+} ϕ=30 nm	Octylamine-PAA ($ϕ_{HD}$ ~ 90nm) Blood circulation half-time = 0.13 / 3.5 min	200 µl, 2 mg mL^{-1} 20 mg kg^{-1} b.w. Injected intravenously through the tail vein	Female Balb/c mice (~20g) Liver :	deposition – ICP of Y^{3+} [% ID g^{-1}] 1, 3,7, 20, 40, 90 days post injection	No obvious histology based organ damage, lesion, serum biochemistry, hematology and	61

			Spleen : 40% at 1st day, ~30→20% afterwards Bone : 230% at 1st day, ~180→120% afterwards Lung : ~10 % Kidney/ Heart/ /Stomach/ 30% at 1st day, <10% afterwards Intestine/ <5% /Skin/ Muscle	histology examinations were observed at 20 mg kg^{-1} dose. UCNP may be partially decomposed or damaged inside macrophage cells without significant excretion.	
β-$NaYF_4$: 20%Yb^{3+}, 2%Tm^{3+} ϕ=30 nm	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG (ϕ_{HD} ~ 100nm) Blood circulation half-time =5.1 / 13.1 min	200 ml, 2 mg mL^{-1} 20 mg kg^{-1} b.w. Injected intravenously through the tail vein	Female Balb/c mice (~20g) deposition – ICP of Y^{3+} [% ID g^{-1}] 1, 3, 7, 20, 40, 90 days post injection Liver : 95% at 1st day, ~50% afterwards Spleen : 240% at 1st day, ~200% afterwards Bone : ~20 % Kidney/ Heart/ /Lung/ Stomach/ Intestine/ Skin/ Muscle : <30% at 1st day, <10% afterwards	No obvious histology based organ damage, lesion, serum biochemistry, hematology and histology examinations were observed at 20 mg kg^{-1} dose. UCNP may be partially decomposed or damaged inside macrophage cells without significant excretion.	61
^{18}F-labeled $NaY_{0.2}Gd_{0.6}Yb_{0.18}Er_{0.02}F_4$ persistent luminescence nanophosphors 22 x 19 nm	citrate-capped	^{18}F-cit-NPs (148 kBq, 1 mg) in 100 mL of saline were injected through the tail vein	mice (18-20 g) Radioactivity in the organs was measured using a γ-counter 15 min and 2 h post-injection 15 min; 2h Liver : 70.8; 46.1 Spleen : 55.7; 75.3 Lung : ~9; ~8 Bone : <3; <3 Heart : ~4; ~3 Kidney : <3; <3 Muscle : <3; <3 Blood : <2; <2	simultaneously possessing with radioactivity, magnetic, and up-conversion luminescent properties	152
$Ca_{0.2}Zn_{0.9}Mg_{0.9}$-Si_2O_6 doped with rare-earth cations (Eu^{3+}, Dy^{3+}, Mn^{3+}), Hydrodynamic diameter, ranging from 80 to 180 nm	poly(ethylene glycol) (PEG)	Nanoparticles (1 mg mL^{-1}) were excited for 5 min under UV light (254 nm). Afterwards a 100 µL portion of a colloidal solution (1 mg PLNP resuspended in 1 mL of sterile 150 mM NaCl) was injected in the tail vein	Five weeks old female Balb/c healthy mice delayed fluorescence from europium ions %ID, 6 h after injection for hydroxyl; 5kDa PEG; 10 kDa PEG and 20 kDa PEG 80 nm NP: hydroxyl 5 kDa; 10 kDa; 15 kDa Liver : 48; 28; 23; 28 Spleen : <2; ~10; ~10; ~10 Kidneys : <3; Lungs : <5 120 nm NP Liver : 100; 30; 31; 55 Spleen : <3; 47; 38; 20 Kidneys : <3	Tissue distribution was found to be highly dependent on surface coverage as well as core diameter. The amount of PLNP in the blood was highly increased for small (d < 80 nm) and stealth particles. On the opposite, PEG shield molecular weight, ranging from 5 to 20 kDa, had only negligible influence on the *in vivo* biodistribution of silicate-based material.	16

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			Lungs : <3		
			180 nm NP		
			Liver : 81; 51; 31; 58		
			Spleen : 29; 63; 72; 49		
			Kidneys : <3		
			Lungs : <5		
			80 nm NP 10 kDa PEG		
			Tumor (6h) 5.9		

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Concluding cited research, UCNPs of various types are usually quickly removed from circulation and deposited in liver and spleen where they can persist for longer time. However no obvious adverse health effects could be detected at doses and experiment times studied. Accumulation of nanoparticles in other organs (mainly heart and lungs) was much lower, rather transient as in most cases nanoparticles continued to deposit in liver and spleen with insignificant remnants at other sites. Tissue distribution has been found to be highly dependent on surface coverage as well as on the core diameter of the nanoparticles. Nanopartcle carrying charged surfaces were rapidly uptaken by liver, presumably by action of opsonins and phagocytic cells. Application of neutral coating ensured by PEG allowed to dramatically increase circulation time in blood. At the same time, differences in molecular weight of PEG used for coating (range from 5 to 20 kDa) had negligible effect on the distribution. Another meaningful factor was the size of used particles, smaller particles showed the longest persistence in circulation. No organ damage or lesions were observed in histological examinations. In addition, at doses up to 20 mg kg^{-1} no changes were observed in the serum biochemistry, and hematology. UCNPs may be partially decomposed or damaged inside macrophage cells without significant excretion, which was concluded from the presence of non luminescent deposits found in UCNPs treated cells using TEM analysis [172, 173].

4.2. *In vitro* studies

Cell cultures incubated with different concentration of silica-coated UCNPs showed a viability rate of approximately 50% even at the highest concentration of 100 µg/mL [150]. PEG-coated UCNPs exerted only minor negative effect on proliferation of human nasopharynx carcinoma (KB) cells. More than 90% of cells survived after incubation for 24 h with 500 µg mL^{-1} of fNPs [17]. Cytotoxicity of UCNPs was also investigated on SK-BR-3 cell lines[174]. The authors concluded that UCNPs were not toxic to the cells because at an injected concentration of below 50 µg mL^{-1} and 80 µg mL^{-1} the cell viability was 98.6% and 92.5% respectively. Chatterjee al. [151] showed the impact of PEI-coated UCNPs on rats and cell culture. These UCNPs showed no toxic effect on bone marrow derived stem cells. Low *in vitro* toxicity of lanthanide doped UCNPs was proven as well by Xing et al. [13]. The HL-7702 and RAW264.7 cells treated with high concentration of $NaYbF_4$ (1.6 mg Yb^{3+} mL^{-1}) for 24 hours survived well – more than 82.7% of HL-7702 and 88.9% of RAW264.7 cells remained viable. The interaction of $NaYF_4$ UCNPs with living cells (HeLa, LO2, KB) were investigated and proved low toxicity of UCNPs [164]. More than 80% of cells survived after incubation with 800 µg mL^{-1} UCNPs for 24 hours.
In 2011, Nam et al. investigated the dynamics of cellular transport of 40 nm PEG–phospholipids coated $NaYF_4$:Yb^{3+}, Er^{3+} UCNPs [175]. Real-time studies of endocytosed UCNPs at the single vesicle level allowed them to conclude that UCNPs were internalized by nonspecific endocytosis since the UCNPs did not have any specific ligand at the surface that could bind to cell receptors. Taking into account the size of PEG-coated UCNPs the most likely mode of endocytosis is pinocytosis. The motor proteins such as dyneins and kinesins are responsible for the active transport of these UCNPs. A toxicological study on PEGylated Yb_2O_3:Er^{3+} upconverting nanoparticles has been also reported [176]. MTT assay on HepG2 cells showed negligible impact of the UCNPs on cell viability (more than 90% cells survived after 12 h incubation with up to 1 mg Yb^{3+} mL^{-1} UCNPs) and on their morphology [176]. Another study on the toxicity of $BaYbF_5$:Tm^{3+}-PAA-nanoparticles also showed low cytotoxicity to the cell culture– the cells proliferated well and there were no morphological changes [18]. ICP-MS analysis of Ba^{2+} and Yb^{3+} ions following long-term dialysis of $BaYbF_5$:Tm^{3+}-PAA at different pH showed the absence of the aforementioned in solution.

Hemmer et al. [189] studied the cytotoxic effect of ligand coated (PEG-b-PAAc) and ligand free Gd_2O_3:Er^{3+},Yb^{3+} nanoparticles. The authors reported cytotoxic effect on phagocytic cells and attributed this to the release of Gd^{3+} ions resulting from the chemical instability of Gd_2O_3 in a low pH environment, which is found in the phagocytic macrophage. Non-phagocytic B-cell hybridoma cells were found to be less prone to cytotoxic effect. The viability of both cell lines improved when they were incubated with PEG-b-PAAc coated Gd_2O_3 nanorods and no effect was observed on the proliferation and morphology of the two cell lines. Long-term dialysis studies of PEG-Gd_2O_3:Eu^{3+} in PBS have been reported [9] to investigate the potential leaching of Gd^{3+} ions. ICP-MS results showed that no free Gd^{3+} ions after 15 days of dialysis. Moreover, PEG-Gd_2O_3:Eu^{3+} was stable in FBS and DMEM cell medium and showed no toxicity towards HepG2 and MCF-7 cells. Spatiotemporal distribution of UCNPs at the single cell level have been conducted by Bae et al. [177]. They used epi-fluorescence imaging techniques to visualize the pathway of UCNPs transport – endocytosis, intracellular active transport, and exocytosis in HeLa cells. This study paves the way to understand the interaction between UCNPs and biological systems. Upconverting $NaYbF_4$:Tm^{3+}@CaF_2 coated with PAA and PEI was successfully used to label mesenchymalstem cells (MSCs) without specific targeting [178]. The authors showed that the UCNPs did not cause cell death and did not negatively impact proliferation of MSCs. In co-culture experiments, no leakage of the internalized UCNPs occurred *via* exocytosis which is important when considering *in vivo* tracking.
The current cytotoxicity studies of the impact of various Ln:NPs and UCNPs on different cell lines are summarised in Table 2. The NPs type, size and surface functionalization as well as cell type and the worst case (*i.e.* highest) NPs concentration and incubation times are presented along with the achieved cell viability for such conditions.
Although the obtained results of impact of UCNPs on living cells can be helpful for predicting and assessing the nano-toxicity, this type of analysis is basically limited to chemical toxicity of NPs themselves or ligands, which are responsible for stabilization and biocompatibility of these NPs. Moreover, these *in-vitro* toxicity tests allow studying the up- and down- regulation of proteins, as well as internalization mechanisms, targeting capability and deposition of NPs in the specific cell's organelles. It would be of great interest to expand these *in-vitro* cellular studies to understand interactions of various NPs with living cells, with special interest of NPs exposed to external factors such as electro-magnetic fields or other types of NPs. In opposite to *in-vitro* cell models, *in-vitro* tissue models are suitable to deliver more information on NPs corona formation, NPs diffusion, aggregation and transportation throughout vessels and tissues of different type as well as long term interactions between the UCNPs and biological constituents. Unfortunately, we are not aware of research performed on such *in-vitro* tissue models, which, in conjunction with conventional *in-vitro* cellular assays, should be of high impact for the nano-toxicity assessment.
For example, it is unclear whether the cell viability decrease or variability of the results between studies upon increased concentration of NPs (as presented in Table 2) result from increased chemical toxicity of NPs or from affecting the transportation of nutrients and gases to the mitochondria, by either limiting their influx or by interaction of these nutrients with NPs' ligands. It appears, that *in-vitro* studies should also include testing cell viability in connection with synergetic response of NPs to factors such as temperature rise, pH variability, exposure to ROS and light etc.. Testing the impact of a much wider array of factors, potentially influencing the UCNPs bioactivity, appears to be inevitable, since it is already known, that biological activities of NPs can be triggered or modulated by small changes in external environment (Fig.3 and Fig.4).

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Table 2. Summary of cytotoxicity studies of various Ln:NPs and UCNPs. The results are presented for the worst case, i.e. highest NP concentrations.

NPs	Size / shape [nm]	Biofunctionalization/ bioconjugation	Cells	Conditions: [NP], incubation time (worst case)	Cell viability (worst case)	Ref.
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ=50 nm	poly(ethylene imine) (PEI)	BMS	1 μg mL^{-1}, 24-48 h 25 μg mL^{-1}, 24-48 h	100% 90%	151
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ=21±0.5 nm	SiO_2	BMS	100 μg mL^{-1}, 24h 100 μg mL^{-1}, 48 h	79.5% 66.8%	170
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ=21±0.5 nm	SiO_2	skeletal myoblasts	100 μg mL^{-1}, 24 h 100 μg mL^{-1}, 48 h	87.8% 68.2%	170
$NaYF_4:Yb^{3+}, Tm^{3+}$	ϕ~11.5 nm	poly(acrylic acid) (PAA)	KB	6-480 μg mL^{-1}, 24 h 480 μg mL^{-1}, 48 h	>94% >80%	156
$NaYF_4:Yb^{3+}, Er^{3+}, Gd^{3+}$	22x19 nm (elliptical shape)	Citrate	KB	500 μg mL^{-1}, 4 h 500 μg mL^{-1}, 24 h	>88% 81%	152
$NaGdF_4:Yb^{3+}, Er^{3+}, Tm^{3+}$	ϕ=25-60 nm	azelaic acid	KB	500 μg mL^{-1}, 4 h 500 μg mL^{-1}, 12 h	90% 90%	171
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ=18-25 nm	Os(II) complex	KB	0, 200, 400 800 μg mL^{-1}, 5h 0, 200, 400 800 μg mL^{-1}, 24 h	>85% >80%	164
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~8 nm	PEG	KB	0, 25, 50, 100, 200, 400, 500 μg mL^{-1}, 8 and 24 h	>85%	17
$NaYF_4$:18%Yb^{3+}, 2%Er^{3+}	ϕ=50 nm	$mSiO_2$	KB	0, 100, 200, 300, 400 μg mL^{-1}, 4 h or 24 h	>85%	153
$NaLuF_4$: Yb^{3+}, Tm^{3+}, and 153Sm^{3+}	ϕ ~25-30 nm	6-AHA	KB	0, 200, 400, 600, 800, 1000 μg mL^{-1}, 24 and 48 h	>85%	165
$NaLuF_4:Yb^{3+}, Tm^{3+}$	ϕ~7.8 nm	Citric acid	KB	0.8 mg mL^{-1} 5h 0.8 mg mL^{-1} 24h	87 % 82 %	179
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ=18-25 nm	Os(II) complex	HeLa	0, 200, 400 800 μg mL^{-1}, 5h 0, 200, 400 800 μg mL^{-1}, 24 h	>85% >80%	164
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~20 nm	6-aminohexanoic acid/folic acid (AHA/FA)	HeLa	400 μg mL^{-1}, 24 h	90%	135
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~10 nm	SiO_2@hypericin-FA	HeLa	0, 25, 50, 100, 200, 400, 800 μg mL^{-1}, 24 h	>90%	154
$NaLuF_4:Gd^{3+}, Yb^{3+}, Er^{3+}/Tm^{3+}$		sodium glutamate or DTPA	HeLa	0, 50, 100, 200, 400, 800μg mL^{-1}, 4 h 0, 50, 100, 200, 400, 800μg mL^{-1}, 24 h	>90% >90%	180
$KGdF_4:Yb^{3+}, Er^{3+}$	ϕ~10 nm	PEI 6AA	HeLa	0-5 mg mL^{-1}, 20 h 0-5 mg mL^{-1}, 20 h	60% >75%	155
$NaYF_4:Yb^{3+}, Tm^{3+}$, $NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~25-30 nm	Cysteine	HeLa	0-300 μg mL^{-1}, 24 h	>97%	139
$NaGdF_4:Yb^{3+}, Er^{3+}$		cyclin D-specific inhibitory peptide	HeLa	50-500 μg mL^{-1}, 24h	>70%	181
$NaYF_4:Yb^{3+}, Tm^{3+}$	ϕ=20-30 nm	oleic acid	Panc 1	2.0 mg mL^{-1}, 10 min	>90%	182
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~30 nm	SiO_2/folic acid (SiO_2/FA)	SK-BR-3	80 μg mL^{-1}, 24 h	92.5%	174
$NaYF_4:Yb^{3+}, Er^{3+}$		SiO_2 (L~12 nm); poly(acrylic acid) (PAA)	Human osteosarcoma cells	1 mg mL^{-1}, 9 days; 1 mg mL^{-1}, 9 days	96.2% 92.8%	183

$NaYF_4:Yb^{3+}, Er^{3+}, Tm^{3+}$	ϕ~14 nm	RGD (arginine-glycine-aspartate tripeptide)	human glioblastoma cell line U87MG	1000 μg mL^{-1}, 24 h	>85%	[184]
$NaYF_4:Yb^{3+}, Tm^{3+}, Gd^{3+}$ $NaYF_4:Yb^{3+},Tm^{3+},Gd^{3+}$@ $NaGdF_4$	ϕ~17.2 nm ϕ~19.3 nm	PEG, PEG/ANG	U87MG	0-1000 μg mL^{-1}, 24 h	>85%	[185]
$NaYbF_4$: 2%Tm^{3+}	ϕ~33 nm	PEG	RAW 264.7 Leukemic monocyte/macrophage	0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg Yb^{3+} mL^{-1}, 24 h	>88.9%	[13]
$NaYbF_4$:2%Tm^{3+}	ϕ~33 nm	PEG	HL-7702	0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg Yb^{3+} mL^{-1}, 24 h	>82.7%	[13]
$NaGdF_4:Yb^{3+}, Er^{3+}$	ϕ~24 nm	dimeric cyclic RGD peptide	U87MG	0 to 200 μg mL^{-1}, 18 h 0 to 200 μg mL^{-1}, 72 h	>95% 80%	[186]
$NaLuF_4:Yb^{3+},Tm^{3+}$	ϕ~7.8 nm	Citric acid	macrophages	0.8 mg mL^{-1} 5h 0.8 mg mL^{-1} 24h	87 % 82 %	[179]
$LaF_3:Yb^{3+}, Ho^{3+}$; $LaF_3:Yb^{3+}, Er^{3+}$	ϕ~15 nm ϕ~15 nm	poly(ethylene glycol)PEG	KB	500 μg mL^{-1}, 4 h 500 μg mL^{-1}, 12 h	>78% >78%	[187]
$Gd(OH)_3$	nanorods 10x200 nm		KB	200, 400, 600, 800, 1000 μg mL^{-1}, 24 h 200, 400, 600, 800, 1000 μg mL^{-1}, 48 h	>90% >85%	[10]
$BaGdF_5:Yb^{3+}, Er^{3+}$	ϕ~12 nm	PEG	HeLa	150, 500, 1000, 2500μg mL^{-1}, 24 h	>86%	[188]
Y_2O_3	ϕ~76 nm	PEG	RAW 264.7 Leukemic monocyte/macrophage	0.5 mg mL^{-1}	>95%	[189]
$Gd_2O_3:Yb^{3+}, Er^{3+}$		bare PEG-b-PAAc	B-cell hybridoma	1 to 100 μg mL^{-1}, 48 h 1 to 100 μg mL^{-1}, 48 h	<60% 90%	[190]
$Gd_2O_3:Yb^{3+}, Er^{3+}$		bare PEG-b-PAAc	HepG2	1 to 100 μg mL^{-1}, 48 h 1 to 100 μg mL^{-1}, 48 h	70% 80%	[190]
$Yb_2O_3:Er^{3+}$		PEG	HepG2	0.01 to 1 mg Yb^{3+} mL^{-1}, 12 h	>90%	[176]
$Gd_2O_3:Yb^{3+}, Er^{3+}$	nanorods (110-180 nm, 15-30 nm)	PEG	HepG2	0 to 1000 μg RE mL^{-1}, 12 h	>95%	[172]
$BaYbF_5:Tm^{3+}$	ϕ~15 nm	PAA	HepG2	200 μg mL^{-1}, 48 h	>90%	[18]
$Gd_2O_3:Er^{3+}$	nanorods (100-150 nm, 10-20 nm)	PEG	MCF-7	0-200 μg mL^{-1}, 48 h	>97%	[9]
$Gd_2O_3:Er^{3+}$	nanorods (100-150 nm, 10-20 nm)	PEG	MCF-7	0-200 μg mL^{-1}, 48 h	>90%	[9]
$Gd_2O_3:Yb^{3+}, Er^{3+}$	nanorods (110-180 nm, 15-30 nm)	PEG	293T	0 to 1000 μg RE mL^{-1}, 12 h	>95%	[172]
$NaYF_4:Yb^{3+}, Er^{3+}$	ϕ~10 nm	SiO_2@hypericin-FA	SW480 human colon cancer cells	0, 25, 50, 100, 200, 400, 800 μg mL^{-1}, 24 h	>90%	[154]
$NaGdF_4:Yb^{3+}, Er^{3+}$@$NaGdF_4$	ϕ~10,5 nm	pSi NPs, pluronic F127 (pSi@UCNPs@F127)	Capan-1 cells	0.001875, 0.00375, 0.0075, .015, 0.03, 0.06 nM, 24 h	>90%	[191]
$NaYbF_4$: Ce^{3+}, Tb^{3+}	ϕ~16.7 nm	MHPC or PEG or Ni-NT	L929 fibroblast	0.5-5000 pM, 48 h	40%	[160]

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$BaGdF_5$:20%Yb^{3+}, 2%Tm^{3+}@ $BaGdF_5$:2% Yb^{3+}	ϕ~10 nm	Gelatin, DOX	BCECs brain capillary endothelial cells	3.125 – 200 μg mL^{-1}, 24 h	>99.2%	[192]
$NaYF_4$:Yb^{3+}, Tm^{3+}, Gd^{3+} $NaYF_4$:Yb^{3+}, Tm^{3+},Gd^{3+}@ $NaGdF_4$	ϕ~17.2 nm ϕ~19.3 nm	PEG, PEG/ANG	A549	0-1000 μg mL^{-1}, 24 h	>85%	[185]
$NaYF_4$:Yb^{3+}, Er^{3+}	ϕ~90 nm		MDA-MB231 negative breast cancer cells	1 mg mL^{-1}, 24 h	>90%	[193]
Gd_2O_2S:5%Eu^{3+}	ϕ~100 nm			0.1; 0.5; 1; and 2 mg mL^{-1}, 24 h or 72 h	>65%	[194]

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The results of the studies, which have been discussed herein, indicate that the cellular viability varies slightly with the ligands used (viability decreased in the order PAA; PEI > AA; AHA; FA; OA > RGD > citrate > PEG > SiO_2), but it is difficult to draw general conclusion, due to different size, doping, shape, crystallographic structure, ligands and composition of nanoparticles used, as well as different incubation times and variety of cells used for studies. SiO_2 coated NPs, have been shown to demonstrate a viability of approximately 68 %, whereas all the other NPs showed a viability above 85%.

One should also mention luminescent nanoparticles that exhibit inherent biocompatibility and may become effective nanolabels. Hydroxyapatites (HAP, $Ca_{10}(PO_4)_6(OH)_2$) are main inorganic component of biological hard tissues such as bones or enamel. HAP have been successfully applied in orthopedic or dental treatments, as bone or titanium implants, non-viral carriers in gene delivery (transfection) and as luminescent labels [195, 196] and luminescent drug carriers [197]. As HAP are stable in biological fluids, they demonstrate good biocompatibility, are porous (so can be used for drug storage and delivery [182, 198]) and biodegradable. The biodegradability is an interesting feature for *in vivo* biological imaging [199], sensing and drug delivery, when toxicity aspects are considered. The HAP NPs can be internalized in living cells, but the control of the size and aggregation is difficult in these NP and may be an obstacle to the development of water soluble biological luminescence nanomarkers [200, 201].

5. Conclusions and final remarks

Notwithstanding the number of studies and existing government reports [202], knowledge on the general impact of engineered nanomaterials on humans health is not well established, thus risk assessment studies and more on hazard and exposure are required. Even the most comprehensive EU chemicals legislation: REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), does not refer specifically to nanomaterials in the document. Regulations are needed, as UCNP studies, which contribute to development of new biomedical methodologies, have significantly expanded and continue to grow tremendously every year.

A significant challenge in predicting the potential toxicity of nanoparticles is due to their complexity. These materials demonstrate biological and environmental behavior that results from a synergistic interaction between their:

- chemical composition (of the NP alone),
- surface state (*i.e.* colloidal stability, NPs uptake and clearance, surface charge/functionality/porosity/ area as well as hydro-/lipo- philicity/phobicity),
- physical parameters (*i.e.* primary and hydrodynamic size, shape, size distribution, structure, agglomeration and concentration)
- local chemical and physical environmental factors (*i.e.* dissolution rate, pH, temperature, corona formation *etc.*)
- presence of other chemicals/biocomponents or electro-magnetic field *etc.*

with the target biological systems [203, 204]. Chemical characterisation of NPs is critical to understand the processes and reactivity of nanoparticles when their surface come in contact with living cells. This knowledge is indispensable for understanding the toxicity of engineered nanoparticles [60, 72]. It is important to mention, that without versatile, profound and well-defined studies on the interaction between NPs and cells, tissues and whole organisms, it will be very difficult to assess and compare the toxicity of various nanomaterials. The reason for this is that conventional toxicological studies rely on results obtained with respect to concentration, composition and time of exposure of the toxic agent. It is clear that nanotoxicology must take into account many more interdependent parameters, and the very basic question, how should the toxic dose be defined, must be answered. It has been postulated, that not the mass, but rather the surface of NPs having been uptaken [68] should be employed to quantify the toxicity of the NPs. This illustrates the level of complexity that nanotechnology faces, trying to evaluate the adverse health and environmental effects.

The majority of the studies, which have been discussed in this review, have shown the UCNPs to have rather low toxicity. However, one cannot conclude at this time that the UCNPs are without health risks. There are no reports on complex long-term (*i.e.* a few animal generations) toxicity of the UCNPs or the reagents and ligands used in the synthesis and (bio)functionalization. Their interaction with other nanoparticles is another challenge that must be addressed. There is also a need to understand how and where the UCNPs accumulate, and what time is indispensible to clear the NPs from the various organs (*e.g.* liver, spleen, lungs). Another important unknowns are, whether the UCNPs decompose (*e.g.* in liver) and contribute to secondary toxicity effects through the interactions of decomposition products with cellular biochemical environment, thus hypothetically leading to cellular response.

There are reports showing that NPs with hydrodynamic diameter larger than 20 nm are not quickly excreted by the urinary system and may circulate in the body for over a week or longer [2, 16, 61]. This is sufficient time for the NPs to interact with bio-components or other NPs. Thus, knowledge of these interactions is paramount in order to shed light on the potential effects that these may have. The size of NPs is also extremely important when considering cellular uptake by endocytosis. Nanoparticles larger than 50 nm are taken in by cells much less efficiently than smaller ones [205], which may reduce the potential toxic effects on cells. Paradoxically, the principle objectives for bio-engineered nanoparticles are to facilitate diffusion, to incorporate highly selective and precise bio-targeting capabilities at the cellular or subcellular levels.

One other important issue concerning the toxicology of the nanoparticles, is the lack of standardized protocol for the assessment of cytotoxicity [6]. It is difficult to compare the results from various studies because the cytotoxicity tests were performed under different conditions (time of exposure and dose) using nanoparticles with varying morphology, chemical composition, size, surface charge or functional groups and on various cell lines [206].

More detailed studies are also required for further evaluation of the toxicity of UCNPs. Although the chemical elements of the UCNPs may themselves be non-toxic, and secondary toxicity is not observed, the UCNPs physical and chemical properties as a whole may cause adverse effects, trigger undesirable cellular signaling and

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cellular distribution [14, 15]. Very small UCNPs can cross the blood-brain barrier, accumulate in various cells or may even penetrate the nuclei [206, 207].

A wide range of applications exploiting the advantages of lanthanide up-converting nanoparticles *in vitro* remain undoubtly highly promising and competitive to traditional approaches. Numerous studies report negligible or low toxicity of lanthanide doped UCNPs which is encouraging future research of UCNPs for biomedical applications. Nevertheless, their use *in vivo* is still questionable, mostly due to the paucity of knowledge concerning primary and secondary toxicity effects on the environment and humans. The lack of systematic fundamental research on the toxicological aspects of UCNPs may inhibit their commercial applications. Therefore, the challenge of assessing the toxicological effects of UCNPs should be a primary concern and at this time all nanostructures should be handled with the same protocols available for handling highly toxic chemicals.

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Abbreviations:

AHA – 6-aminohexanoic acid
ALT – alanine aminotransferase
APTE - (fr.) Addition de Photons par Transferts d'Energie
AST – aspartate aminotransferase
AuNPs – gold nanoparticles
CA – contrast agent
CNT – carbon nanotube
DOX – doxorubicin
DWCNT – double-wall carbon nanotube
FA – folic acid
fNPs – functionalized nanoparticles
FRET - Förster resonance energy transfer or fluorescence resonance energy transfer
GGT – glutamyltranspeptidase
GI – gastrointestinal
GLO – globulin
HAP – hydroxyapatite
ICP – Ion Coupled Plasma
ID – intradermal
IM – intramuscular
IP – intraperitoneal
IV – intravenous
LD – lethal dose
LD50 – lethal dose leading to death of half of the tested animal population
MPS – mononuclear phagocyte systems
MRI - magnetic resonance imaging
MWCNT – multi-wall carbon nanotube
NADH - nicotinamide adenine dinucleotide
NIR - near-infrared
NC, NP - nanocrystal, nanoparticle
PAA – poly(acrylic acid)
PBS – phosphate buffer saline
PEG – poly(ethylene glycol)
PEI - poly(ethylene imine)
PDT – photodynamic therapy
PO – per os
PVP – polyvinylpyrrolidone
RGD - arginine-glycine-aspartate tripeptide
QD – quantum dot
ROS – reactive oxygen species
SNR – signal to noise ratio
SP-A – surfactant protein A
TBA – total bile acid
TBIL – total bilirubin
UC – up-conversion
UCI - up-conversion imaging
UCL – up-converting luminescence
UCNPs – up-converting nanoparticle

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