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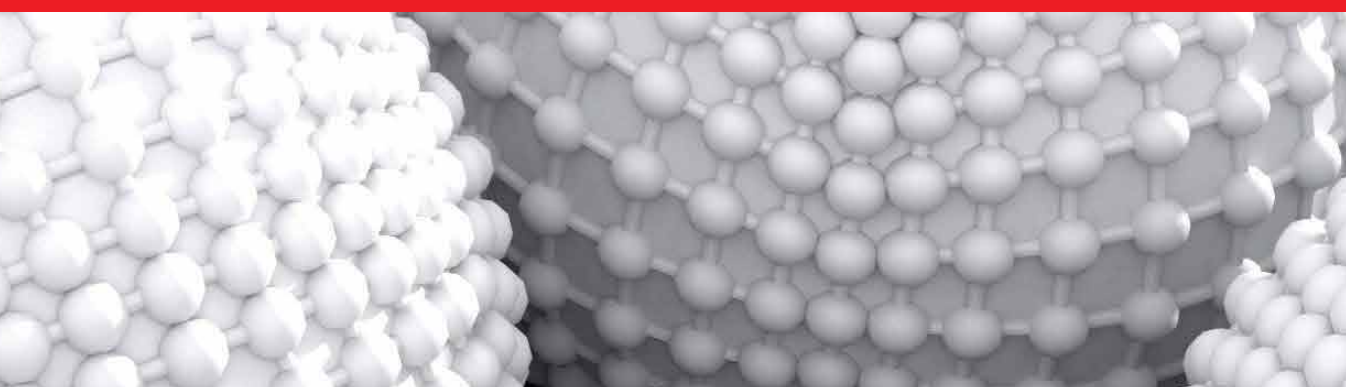
Toxicity of Nanoparticles

Recent Advances and New Perspectives

Edited by Mohammed Muzibur Rahman, Jamal Uddin, Abdullah Mohamed Asiri and Md Rezaur Rahman



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Contents

Preface **XI**

Section 1
State-of-Art Nanotechnology **1**

Chapter 1 **3**
Toxicity of Quantum Dots
by Gerardo González De la Cruz, Lourdes Rodríguez-Fragoso, Patricia Rodríguez-Fragoso and Anahi Rodríguez-López

Chapter 2 **17**
Nanotoxicological Assessments of Upconversion Nanoparticles
by Dalia Chávez-García and Karla Juarez-Moreno

Chapter 3 **37**
Toxicity of 2D Materials and Their Future Prospect
by Subash Adhikari

Chapter 4 **55**
Potential Toxicity of Nanoparticles for the Oral Delivery of Therapeutics
by Iman M. Alfagih

Section 2
Chemical State-of-the-Art **77**

Chapter 5 **79**
Phyto-Metallic Nanoparticles: Biosynthesis, Mechanism, Therapeutics, and Cytotoxicity
by Akeem Omolaja Akinfenwa and Ahmed Abdelfattah Hussein

Chapter 6 **105**
Efficacy of Cobalt-Incorporated Mesoporous Silica towards Photodegradation of Azodyes and Its Kinetic Study for Advanced Application
by Krishna Vaddadi, Nookaraju Muralasetti and Naginami Naidu

Chapter 7 **121**
Iron Oxide Nanoparticles: A Mighty Pioneering Diagnostic Tool But Is It Really Safe for Carcinoma and Neurodegenerative Diseases?
by V. Sandhiya, Selvaraja Elumalai, K. Dhunmati, C.N. Nalini, Ganesh Mani, Senthilkumar Balakrishnan and Ubaidulla Uthumansha

Section 3
Biological State-of-the-Art **137**

Chapter 8 **139**
In Vitro, *In Vivo* and *Ex Vivo* Models for Toxicity Evaluation of Nanoparticles: Advantages and Disadvantages
by Neeraja Revi, Oluwatosin D. Oladejo and Divya Bijukumar

Chapter 9 **173**
Biological Agents for the Synthesis of Silver Nanoparticles and Their Applications
by Krishna Gudikandula, Trimothi Dasari and Reeja Sundaram

Chapter 10 **193**
Bioengineered Nanoparticle and Environmental Particulate Matter Toxicity: Mechanisms, Regulations and Applications
by Hemant Sarin

Preface

Toxicity of Nanoparticles – Recent Advances and New Perspectives focuses on the development of nanoparticles for advanced applications. This book reviews the state of the art of various nanoparticles, their facile synthesis, detailed characterizations, and potential applications in toxicological purposes including drug delivery, bioengineering, microbiology, seafood and aquacultures, phytometallics, nanotoxicological assessment, and immunotoxicity. It covers nanoscale aspects of the synthesis, growth, and development of spherical nanoparticles as well as new paths and emerging frontiers in nanoparticle toxicity. The discussion includes fundamentals and conventional applied experimental routes of toxicity, the interaction of nanoparticles in biological and chemical aspects, and the interface of nanoscience and nanotechnology.

This book seeks to bridge the gap between undergraduate, graduate, and research study in advanced nanoscale materials, to introduce scientists to the opportunities offered by applied science and technology. I hope that this contribution will bring new entrants into the fields of applied material science and nanotechnology and help scientists to develop their specializations.

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Section 1

State-of-Art Nanotechnology

Chapter 1

Toxicity of Quantum Dots

Gerardo González De la Cruz, Lourdes Rodríguez-Fragoso, Patricia Rodríguez-Fragoso and Anahi Rodríguez-López

Abstract

Quantum dots (QD) have been deeply studied due to their physicochemical and optical properties with important advantages of a wide range biomedical applications. Nevertheless, concern prevails about its toxic effects, mainly in those QD whose core contains cadmium. Therefore, there are reports about the toxicity caused by the release of ions of cadmium and the effects related to its tiny nanometric size. The aim of this chapter is to show the evaluations about the toxicity of QD, which include studies on viability, proliferation, uptake, and distribution *in vitro* and *in vivo* models. What are the worrying toxic effects of QD? There are reports about some mechanisms of toxicity caused by QD, such as immunological toxicity, cell death (apoptosis and necrosis), genotoxicity, among others. In addition, we discuss how coating QD with passivating agents that improve their biocompatibility. Likewise, this coating modifies their size and surface charge, which are fundamental aspects of the interaction with other biomolecules. We consider highlighting information about more precise techniques and methodologies that help us to understand how QD induce damage in several biological systems.

Keywords: quantum dots, cytotoxicity, cadmium, nanotoxicity, biocompatibility

1. Introduction

In recent decades, there have been countless publications on the use of nanomaterials, particularly in the biomedical area. The main use of semiconductor nanoparticles (NPs) lies in the development of formulations for the delivery of anticancer therapies, specifically targeting diseased tissues and organs. Moreover, quantum dots (QDs) provide remarkable specificity while avoiding damage to surrounding healthy cells and thus avoiding the dreaded side and adverse effects of current treatments. However, among the great applications and their attractive physicochemical and optical properties are a myriad of toxicological effects in biological systems [1]. QDs are inorganic semiconductors with a size range of 1–10 nm. Unlike other types of nanomaterials (NMs), QDs possess a unique and exceptional luminescent property. QDs have become the focus of a study by many researchers [2]. So far, QDs are the most promising option that have exhibited potential for applications in bioimaging (luminescence detection) [3, 4].

Quantum dots have properties, such as luminescent intensity, broad emission spectrum, tight size control, and selectivity, based on their composition. In addition, quantum dots have high resistance to photobleaching, physicochemical robustness, and better half-life than other conventional fluorochromes [5–9]. These

nanomaterials are constituted by central semiconductor core consisting of elements from groups II, VI, III-V, or IV-VI of the periodic table and mostly can be composed of heavy metals and toxic materials (e.g., Cd, Te and Hg, CdS, CdTe, CdSe, among others) [10, 11]. Because their main component is cadmium and because of their tiny size they imply a potential hazard, especially for medical applications. There are different types of cadmium-free quantum dots, such as InP/ZnS, CuInS2/ZnS, AgInS2/ZnS, silicon, and graphene. Although they are cadmium-free in their composition, they are still subject to rigorous toxicological studies [12].

In order to reduce the cytotoxicity of quantum dots, there are some strategies such as the use of some shells composed of ZnS, CdS, ZnSe, or even CdS/ZnS multishells. By covering the core not only improved luminescent effect but also reducing the toxicity by avoiding the release of heavy metal ions [13, 14]. Achieving functionalization of the QD core shell with a polymeric shell can give the desired biocompatibility and decrease its cytotoxic effects [15, 16]. Among some functionalized QDs, there are those coated with polymers such as dextrin or maltodextrin, which make the semiconductor able to target organs and can even be taken up by cellular organelles [17–19]. This advantage allows QD to be more specific and selective for applications for disease diagnosis and treatment purposes. However, the negative effects that QDs may have on cells are difficult to assess. QDs have higher fluorescence intensity, prolonged lifetime, specificity, and possess optical stability compared to conventional fluorochromes. In addition, the wavelength at which they emit is given by tight control of the core size. **Figure 1** shows the characteristic image of QDs emitting photoluminescence.

The characteristics of QDs include size, which is what determines the wavelength at which they emit, although in some cases it does not depend on their composition. Thus, QDs of smaller size (2 nm) emit in blue, QDs of 3–5 nm in green, 6–8 nm in orange, and sizes of approximately 8–10 nm in red [10]. The controversial mechanisms by which QDs are introduced into cells are of great interest among the scientific community and thus the molecular and physiological basis of cytotoxicity. These cytotoxic effects have been classified into *in vivo* and *in vitro*. Thus, cell culture-based tests have become the first choice for bioassessment of QD toxicity [20]. However, *in vitro* studies include assessments of cell membrane integrity, morphological changes, organelle dysfunction, and in some cases quantification of viable cells. Nevertheless,

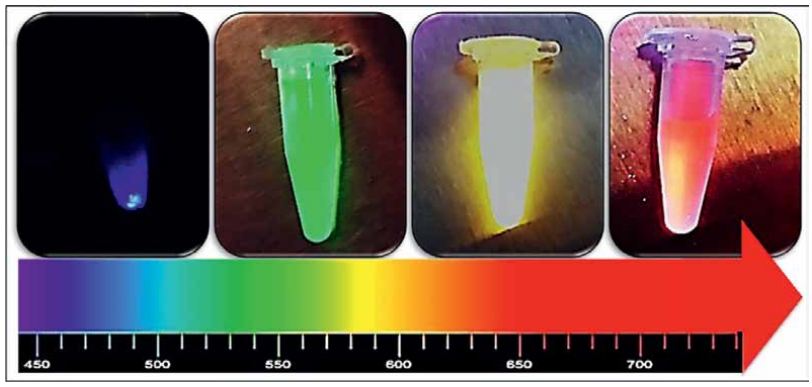

Figure 1.
Fluorescence image of cadmium QD. La emisión de fluorescencia es dependiente del tamaño de los QD. Por lo que, la fotoluminiscencia va del Azul Para aquellos QD más pequeños y hasta el rojo Para los de un núcleo mayor.

information on the behavior of quantum dots in a biological system is still scarce and does not emphasize the cell type-dependent toxicity induced by quantum dots. In this review, we have summarized the efforts in achieving a less toxic design, its advantages and disadvantages in the synthesis of single and bioconjugated quantum dots for application as nanovehicles.

2. Cytotoxic effects of quantum dots on diverse cell lines

The cell membrane is the first barrier that divides intracellular from extracellular mechanisms. The process by which QDs enter the cell is not well defined, although it includes anchoring of QDs to the cell membrane, transmembrane transport, distribution and localization within subcellular compartments, and intracellular accumulation. All these processes are linked to their future application, their potential toxic effects, and the adverse effects induced in a dose-time-dependent manner [21]. Tests such as i*n vitro* cytotoxicity are important because of the significant morphological changes caused by QDs at the cellular and subcellular levels. In recent years, a huge variety of *in vitro* studies suggest that QDs have toxic effects on cells at different levels [22, 23]. In addition, the passage of QDs across the cell membrane has been demonstrated, the effects are oxidative stress, direct damage to membrane, morphological alterations, and various types of cell death.

In vitro models are necessary for safety assessment in preclinical testing of nanomaterials for diagnostic purposes. Although some models for cytotoxicity are not sufficient due to lack of human cells available for culture or even lack of reproducibility in assays. Therefore, the predictability about the safety of a nanodrug is a difficult task for nanotoxicology researchers [24]. However, there are *in vitro* models considered as standard patterns for toxicological studies of nanomedicines such as the use of human renal Hek293 cells [25]. Over a decade, our research group has focused its interest on the study of dextrin-coated 3.5 nm sized cadmium sulfide QDs (CdS-dex) [26] and their potential biomedical application as is the case of doxorubicin-conjugated CdS-dex QDs (CdS-dex/dox) [27]. Therefore, we have established several *in vitro* tests using Hek293, HeLa (cervix adenocarcinoma), and HepG2 (hepatic cells) cells for preclinical studies on CdS-dextrin quantum dots and with maltodextrin. Therefore, our results demonstrate that CdS-dex QDs and CdS-dex/dox QDs induce exposure to dose-dependent cytotoxic effects. In addition to this, we consider that one of the main evaluations to be performed on QDs is the monitoring of their cellular uptake and distribution. We observed that Hek293, HeLa and HepG2 cells when being treated with concentrations of 0.01 and 1 μg/mL, CdS-dex QDs cross the cell membrane, induce morphological changes, and distribute uniformly at different cellular level. Due to their nanometer size, QDs caused cytotoxicity in the three different cell types by crossing the cell membrane. However, morphological changes varied significantly between Hek293, HepG2, and HeLa cells and the concentration of CdS-dex QDs (**Figure 2**). When QDs have contact with the extracellular membrane, they interact with components of the plasma membrane which allows them to somehow enter the cell by some mechanism such as endocytosis. Endocytosis engulfs the QDs by invagination of the membrane to form endocytic vesicles, which transport the QDs to subcellular compartments. Depending on the cell type, as well as some biomolecules involved in the process, endocytosis can occur in different types [28, 29]. Some authors refer to the uncertainty about the toxic effect that quantum dots may cause as they are transported through the bloodstream and leach into the kidneys.

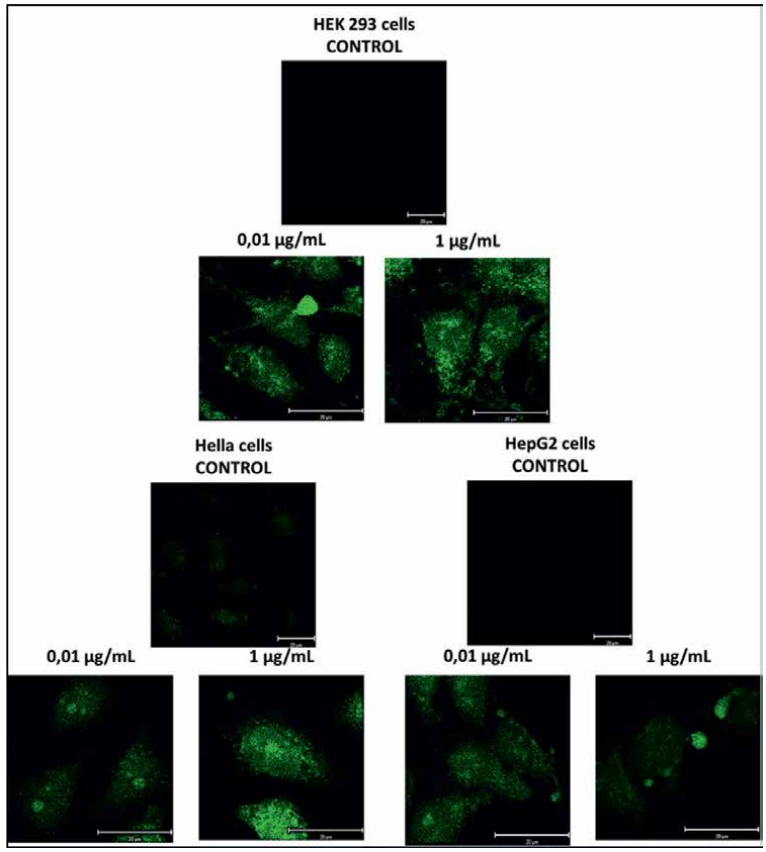

Figure 2.
Fluorescent microscopic visualization of CdS-dex QD in human cell lines. Cells were treated for 24 h with CdS-dex QD (0,01–1 µg/mL). Cells were seeded on slides by smearing and allowed to dry, then analyzed using confocal epifluorescence microscope. Green fluorescence shows the presence of QD surrounding the cytoplasm of Hek293, HeLa, and HepG2 cells. Scale bar 20 µm.

However, there is no information on the nephrotoxic effects of quantum dots both *in vitro* and *in vivo*. Nevertheless, some studies aim to understand the cytotoxic effect on renal cells caused by quantum dots. Therefore, quantum dots, such as titanium oxide (TiO_2), zinc oxide (ZnO), and cadmium sulfide (CdS), have been evaluated in tubular cells (HK-2) in which the cellular and molecular mechanism through oxidative stress induced by quantum dots was demonstrated. In which it was observed that the cytotoxicity of quantum dots was size and solubility dependent. Furthermore, quantum dots that were soluble such as CdS and ZnO were found to cause dose-dependent cell death and degradation/discharge of their ions, respectively [30].

In another investigation, carboxylated CdTe QDs were used and the induced cytotoxicity was evaluated in HeLa cells treated at concentrations from 0.1 to 1000 ng/mL during different exposure times. The effect of CdTe QDs on cell death type, genotoxic effect, and cellular uptake was also evaluated. In this study, they demonstrated that carboxylated QDs did not prove to be less cytotoxic compared to CdTe alone in a concentration-dependent manner. Furthermore, they concluded that CdTe-COOH QDs have genotoxic properties and antiproliferative effects in HeLa cells [31].

Although CdS-dex quantum dots produced different cytotoxic effects on human tumor cells, these effects are not necessarily benign. In fact, our study showed that these nanoparticles had the ability to enter even subcellular compartments. Thus, their biological behavior could trigger pathophysiological effects in a concentration-dependent intrinsic manner. Our CdS quantum dots are coated with a polymeric layer of dextrin. However, many nanomaterials are known to have an inorganic or polymer layer protecting the core to prevent degradation. Even so, heavy metal ions such as cadmium can be released through low stability [32–34]. Studies are needed to know if the cadmium core degrades and releases metal ions and what effects are related to this degradation.

Despite the remarkable effects caused by CdS-dex quantum dots, we clearly need to reinforce the studies and strategies that allow us to learn more about their toxicity. We are getting closer and closer to obtaining biocompatible semiconductor nanoparticles with useful capabilities in diagnosis, treatment, and monitoring of pathologies such as cancer.

Evidently, QDs have physicochemical properties and capabilities and characteristics similar to biological molecules that allow them to be used in biodiagnostics, bioimaging, and targeted drug delivery. For a drug to be effectively delivered using nanocarriers such as QDs, the core component of the QD, the drug or molecule with which it will bioconjugate, and the core shell must be considered. That is, this set of components must be carefully selected to have therapeutic efficiency and optimal safety for use in a biological system [35, 36]. Currently, QDs are considered a tool with promising uses and applications in nanomedicine. However, their cytotoxic effects remain among the main challenges regarding their biocompatibility. The QDs with the highest capacity to emit luminescence and with the highest efficiency in carrying molecules with active principle are those containing cadmium (Cd). However, one of the limitations for the use of Cd QDs in nanomedicine and clinical research is that it is suggested that the core disintegrates and is potentially toxic. That is, it has been considered that it is the core of the QD that largely determines the cytotoxic response and pathophysiological effects [37–39].

Some authors refer that the safety assessment of QDs alone or conjugated is of vital importance since it will allow predicting the effects when interacting with a biological system. They suggest that a nanomaterial is small enough to enter a cell and its cellular compartments, regardless of the route of administration [40–42]. For systemic drug delivery, the intravenous (IV) route is used, which is a major challenge in the development of nanotherapies [43]. The US Food and Drugs Administration (FDA) has approved NMs that have been studied in rigorous preclinical studies combining therapeutic and biological targets as drug delivery agents [44–46].

Our working group has been given the task of synthesizing colloidal CdS-dex/dox QD and evaluating on HeLa cell. We treated HeLa cells with CdS-dex and CdS-dex/dox to compare the selectividad of uptake alone as well as bioconjugated (1 μg/mL) in both cases and with doxorubicin at the same concentration. After 24 h of incubation and in order to investigate the cellular absortion of QD, cells were fixed on slides for visualization by confocal fluorescence microscopy. Through visualization of fluorescence and cellular uptake, we can observe that in cells treated with CdS-dex QDs without bioconjugation, there was a higher distribution in cytoplasm, nucleus, and nucleoli of the cell. However, this cellular uptake and distribution were not the same in the case of HeLa cells treated with doxorubicin and CdS-dex/dox. Nevertheless, in cells treated with doxorubicin and CdS-dex/dox, a significant increase in cell size was observed compared to cells treated with QDs alone. Although, QDs did not appear

homogeneous throughout the cytoplasm and with lower fluorescence intensity in the nucleus (**Figure 3**). They can also induce not only cytotoxic but also genotoxic effects in both normal and cancer cells [47–50].

Although, it has been shown that the effect after cellular uptake of various QDs depends on their size, shape, concentration, and cell type. The cytotoxic effect and mechanisms of nanotoxicity by the interaction of QDs with cells remain complex to assess and far from fully understood. However, this nanotoxicity has been shown to occur intracellularly or extracellularly [51]. QDs can even interact directly with biomolecules once inside the cell, due to their minute size. As a result of this interaction, an alteration in cellular equilibrium coexists, as well as irreversible morphological

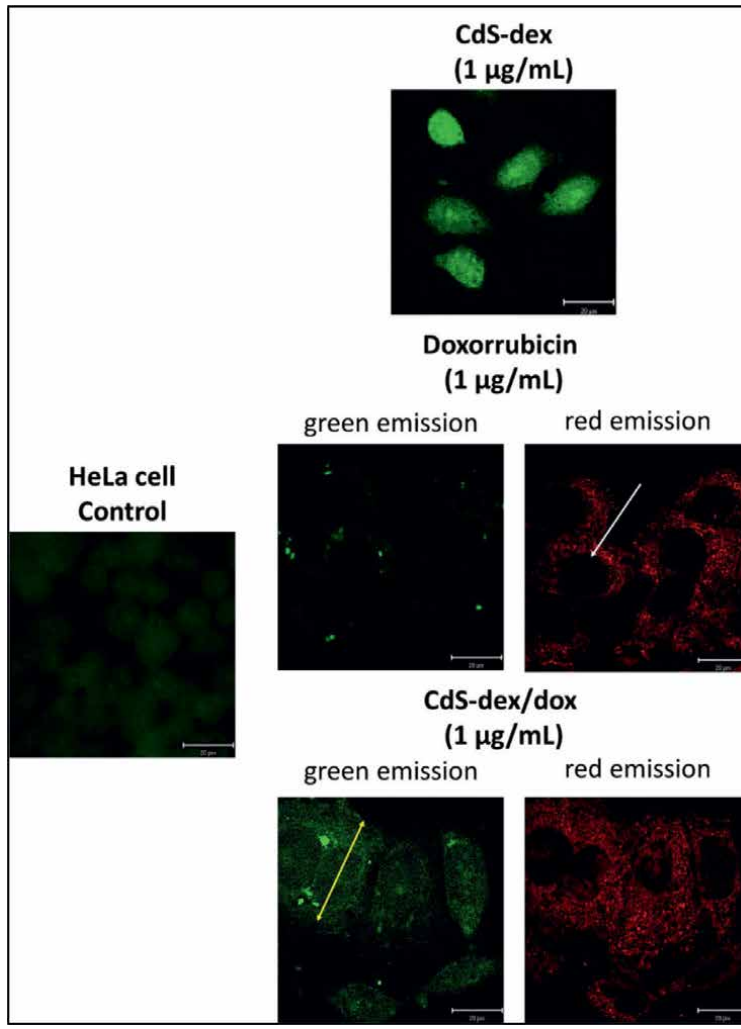

Figure 3.
Fluorescent microscopic visualization of doxorubicin, CdS-dex, and CdS-dex/dox QD in HeLa cell. Cells were treated for 24 h at 0,01–1 µg/mL concentration of doxorubicin, CdS-dex, and CdS-dex/dox QD. Cells were seeded on slides by smearing and allowed to dry, then analyzed using a confocal epifluorescence microscope. Green fluorescence shows the presence of CdS-dex QD. Red emission shows fluorescence in the presence of doxorubicin and CdS-dex/dox QD. The yellow arrow represents the increase in size and the white arrow indicates the absence of QD.

and functional damage [51]. Even if indirectly the outside of the interacts with QDs through membrane receptors that cause activation and inhibition of different signaling pathways, causing toxic reactions or cell death [52].

Therefore, the cytotoxicity of QDs is more complex than we can imagine, it can cause not only the interaction with heavy metals contained in QDs but the disintegration of the core and the release of Cd ions, which increases their toxic potential. Under this condition, researchers have expressed concern about the use of NM and the parameters to be evaluated for future medical applications. This question arises from the association of adverse effects derived from the ability of QDs to enter cells and lodge in various subcellular compartments. This implies that they could evade the defense mechanisms of the human body, cross biological barriers and even interact with components of blood circulation [53]. Moreover, the blood circulation is the primary passage of NMs to the distribution of target organs. Thus, vascular endothelial cells serve as the first barrier and are tasked with maintaining vascular integrity [54]. In a study with ZnO nanoparticles, it has been shown that they are capable of causing cytotoxicity in HUVEC cells due to the increase of intracellular reactive oxygen species (ROS) in a dose-dependent manner [55]. Our studies have shown that at concentrations of 0.01 μg/mL, CdS-dex QDs already cause cytotoxic effects in HUVEC cells. The QDs are distributed around the cytoplasm, producing an increase in cell size and completely changing the characteristic morphology of the endothelial cell (**Figure 4**). Although it does not penetrate into the nucleus and nucleoli, cellular uptake occurs in a dose-dependent manner. In addition, endosome formation is observed, suggesting that cell deformation and toxicity are caused by cellular stress following the passage of the QD into the cell. The cytotoxicity produced by QDs is the

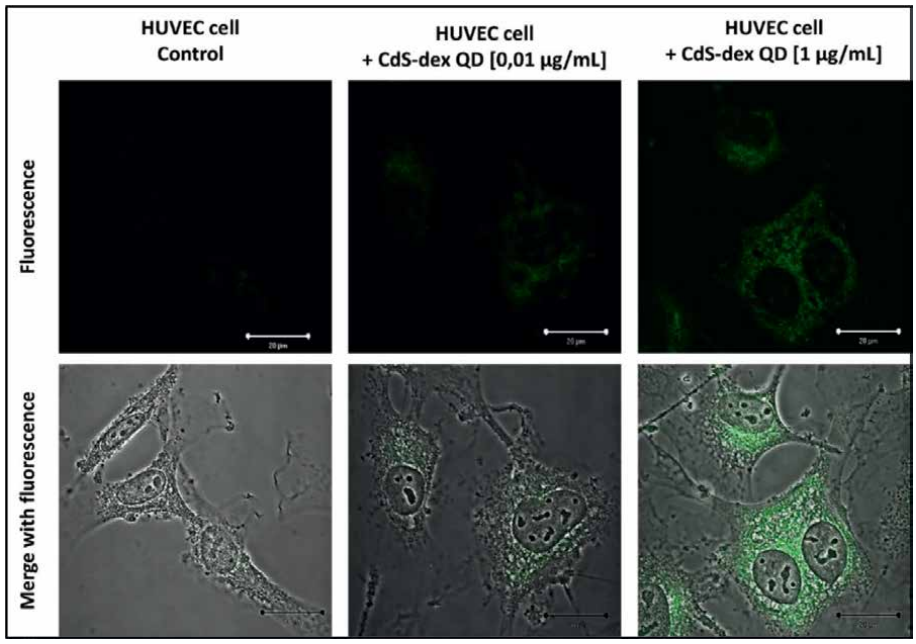

Figure 4.
Fluorescent microscopic visualization of HUVEC cells treated with CdS-dex QD at 0,01–1 μg/mL concentration and 24 h time exposure. Cells were seeded on slides by smearing and allowed to dry, then analyzed using a confocal epifluorescence microscope. Green fluorescence shows the presence of CdS-dex QD.

main parameter limiting their use in bioimaging research. The idea of applying QDs that produce morphological changes and ultimately cell death is a determining factor. Currently, joint efforts are being made for the development of innovative QDs capable of meeting the needs in healthcare areas. This progress in QD design and synthesis has resulted in improved safety *in vitro* studies. However, a myriad of factors that lead to cytotoxicity of QDs in normal, cancer, and endothelial cells remain in question. It has also been demonstrated that when QDs come into contact with organisms, they produce toxicity that is size-dependent, concentration threshold-dependent, and varies according to cytosensitivity [56]. However, factors such as concentration range are responsible for the intracellular distribution, which necessitates storage and bioaccumulation and thus increases cytotoxicity [57]. There is still a long way to go to achieve an accurate understanding and standardized parameters on safety for the use of quantum dots in the field of biomedicine.

In a whole decade, we have been dedicated to the design, synthesis, and nanotoxicological evaluation of quantum dots so we are very clear that, quantum dots can be improved in their design and composition. In addition, the nanoparticle size must be strictly controlled as it is one of the main factors influencing the toxicological effects of quantum dots [53]. The idea of having a complete profile of a type of nanomaterial is not unrealistic. However, it is necessary to demonstrate with studies on its preclinical evaluation. These evaluations include physicochemical characterization, *in vitro* evaluations with different types of human tumor and healthy cells, biodistribution, bioaccumulation, and pharmacokinetic studies. In addition, to perform exhaustive evaluations on its hemocompatibility as a starting point to rule out the toxic effect of a nanomaterial.

3. Conclusion

The development of newer drug delivery systems based on the use of quantum dots is one of the advantages for various disease treatments, such as cancer and gene therapy, as noted above. This modality allows for site-specific drug therapies and a higher safety profile. However, the pharmaceutical industry is far from knowing everything about the toxicological profile of all nanomaterials. However, nanotechnological challenges are evolving and it is necessary to focus our attention on the standardization of parameters for the evaluation of the cytotoxicity of nanomaterials such as quantum dots in order to broaden their safety range and thus ensure lower toxic effects. In the meantime, let us not forget that the key to the toxicity caused by quantum dots is given by the interaction of the elements that compose them and the biomolecules of the biological system. In the very near future, we can include scientific bases that tell us about physicochemical perspectives of quantum dots, better experimental conditions already standardized and reliable comparative analyses (*in vitro* and *in vivo*).

Appendices and nomenclature

NP	nanoparticles
QD	quantum dots
NM	nanomaterials
Cd	cadmium

Te	tellurium
Hg	mercury
CdS	cadmium sulfide
CdTe	cadmium telluride
CdSe	cadmium selenide
CdS-dex	sulfuro de cadmio core/capped dextrina
CdS-dex/dox	sulfuro de cadmio core/capped dextrina, with doxorubicin
HUVEC	umbilical cordon human cells
HepG2	hepatic cells
Hek293	kidney human cells
HeLa	adenocarcinoma of cervix
μg	micrograms
mL	milliliters

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Chapter 2

Nanotoxicological Assessments of Upconversion Nanoparticles

Dalia Chávez-García and Karla Juarez-Moreno

Abstract

Upconversion nanoparticles (UCNPs) are highly efficient luminescent nanomaterials with emission in the visible spectra while being excited by near-infrared region light (NIR). With their unique properties such as high luminescence intensity, sharp emission peaks with narrow bandwidth, large anti-Stokes' shift, and sizes smaller than 100 nm, UCNPs have emerged as promising candidates for diverse biomedical applications such as cancer detection and therapy, fluorescence imaging, magnetic resonance imaging (MRI), and drug delivery. The UCNPs are composed of a crystalline matrix doped with lanthanide ions that can absorb NIR light (~980 nm) and upconvert it to visible light. However, to achieve successful biomedical applications, proper functionalization, target-specific cell interaction, and biocompatibility are critical factors that must be considered. Additionally, a comprehensive nanotoxicological assessment is necessary to ensure that UCNPs are not cytotoxic or genotoxic. This assessment is particularly important for long-term studies of nanoparticles' tracking *in vivo*. Therefore, this chapter aims to provide an in-depth evaluation of the nanotoxicological issues related to nanoparticles (NPs) and UCNPs in biomedical applications, and ensure their safety and efficacy as bioimaging and chemotherapeutic delivery tools.

Keywords: cytotoxicity, nanoparticles, upconversion, nanotoxicological, luminescent

1. Introduction

The toxicity assessment of nanoparticles (NPs) is a relevant issue since many researchers are using, specially, luminescent nanoparticles for various applications, such as bioimaging or drug delivery for *in vivo* and *in vitro* applications [1–3]. In this chapter, we will analyze how the approach in this analysis has been carried out for upconversion luminescent nanoparticles (UCNPs), which are a special type of NPs since they can receive energy in the near-infrared region (NIR) and emit in the visible or NIR spectrum. These NPs are composed of a matrix cell that can be made of oxides, oxysulfides, oxyhalides, phosphates, molybdates, tungstates, gallates, vanadates, and fluorides. The UCNPs are doped with lanthanide elements such as: Yb^{3+}, Er^{3+}, Tm^{3+}, and Ho^{3+}, among others. It is common in the upconversion process to have lanthanide elements co-doped to bring about a photon transfer between energy levels. For example, for the doping of Yb/Er, the Yb^{3+} absorbs NIR radiation at 970–980 nm of

wavelength in its base state ($^2F_{7/2}$–$^2F_{5/2}$), then this energy is transferred to Er^{3+} and the electron is populated to level $^4I_{11/2}$, then, a second photon is absorbed and by Yb^{3+} and it is transferred to Er^{3+}, so the electron is raised to level $^4F_{7/2}$. From this state, it decays rapidly to $^4S_{3/2}$, and the green emission happens ($^4S_{3/2}$–$^4I_{15/2}$), and this process is called the APTE (Addition de photons par transfert d′énergie, i.e., photon energies by adding transfers), as can be seen in **Figure 1**. There are more upconversion processes with different doping combinations and concentrations of the ions, where the percentage of doping directly affects the color of emission [4].

The UCNPs have emerged as a promising nanomaterial for identifying specific cells and for drug delivery. Unlike other dyes, UCNPs exhibit stable emission if the source of excitation is maintained, making them more reliable. There are other types of upconversion processes such as: two-step absorption, cooperative sensitization, cooperative luminescence, the second harmonic generation, and two-photon absorption [4].

One crucial aspect of using UCNPs in biomedical applications lies in ensuring their biocompatibility on cells and or organisms. To achieve this, UCNPs must be functionalized with different ligands that specifically target the desired cells and organs. Several chemical groups, including polyethylene glycol (PEG) [5], polyethyleneimine (PEI) [6], polyvinylpyrrolidone (PVP) [7], polyacrylic acid (PAA) [8], and silica [6], have been used for this purpose. However, it is important to highlight that the toxicology of UCNPs depends on their physicochemical and physiological properties. Physicochemical properties include size, shape, surface area, and chemical composition, while physiological properties refer to the disease conditions, genetics, and other factors [9]. The recommended size for optimal penetration of NPs is below 100 nm. However, this size may also pose a risk of toxicity due to their potential to penetrate cellular structures and organs via the circulatory system. Moreover, UCNPs may generate reactive oxygen species (ROS) that can induce DNA damage, which not only affects the cell growth by means of protein oxidation, but also impacts mitochondrial respiration [10].

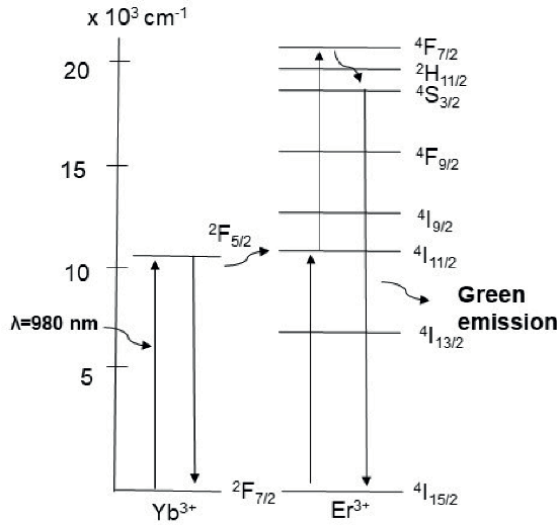

Figure 1.
Upconversion process between Yb^{3+} and Er^{3+} ions.

Several toxicological studies have been conducted on both *in vivo* and *in vitro* human cell lines and organs to assess the potential harmful effects of UCNPs. These studies have evaluated the effects of gene expression, growth, and reproduction of the organisms. It is crucial to continue monitoring and evaluating the toxicity of UCNPs as their use becomes more prevalent in biomedical applications.

2. Biocompatibility of nanoparticles

This section will provide an overview of different methods that researchers use to achieve biocompatibility of UCNPs. **Figure 2** depicts the common way to coat and functionalize UCNPs for several researches, as generally, the UCNPs or NPs need to be coated to ensure biocompatibility and they need functional groups to attach to several types of ligands that can bind to the surface of the targeted cells, as depicted.

2.1 Polyethylene glycol

The most used method to achieve biocompatibility is through PEGylation, which is both effective and straightforward. Although the specific approach may vary among different authors, PEGylation generally refers to the covalent conjugation of PEG to other molecules. This process enhances the physicochemical properties of the molecules, leading to reduce the immunogenicity and improve solubility, electrostatic binding, and hydrophobicity of a given biomolecule [11]. Overall, PEGylation represents a valuable tool for improving the biocompatibility of drugs and biomolecules, allowing for safer and more effective biomedical applications.

The first polymer conjugation was developed by Abuchowski et al. in 1977 [12], and various authors have developed different PEGylation methods for diverse applications, ranging from biocompatibility to trimodal fluorescence. For instance, Zeng et al. [13] developed PEG-modified $BaGdF_5$:Yb/Er UCNPs for multimodal fluorescence/CT (computed X-ray tomography)/magnetic bioimaging applications, which exhibited low cytotoxicity and long circulation time. Similarly, Maldiney et al. [14]

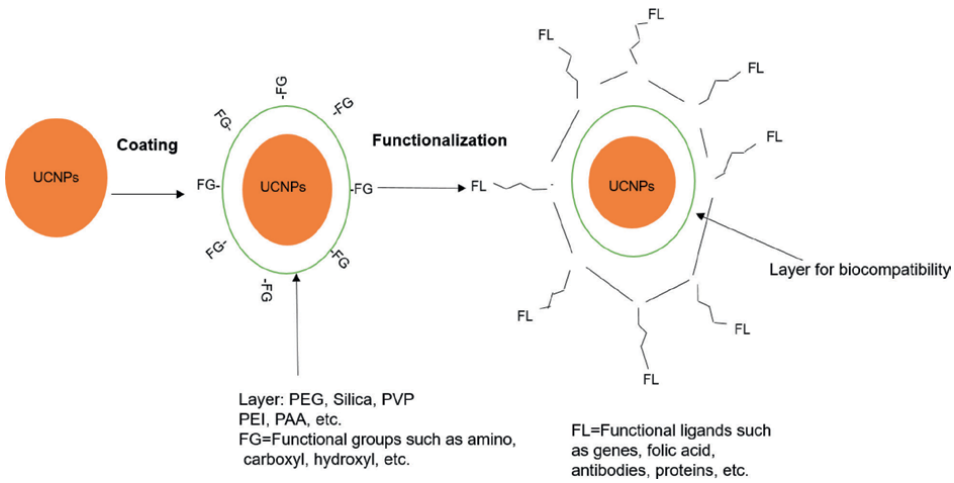

Figure 2.
Biocompatibility and functionalization of several types of UCNPs.

utilized luminescent NPs emitting in the near-infrared spectra, with two types of mice: healthy and tumor carrier mice. They reported that PEG coating enabled the formation of stealthy particles that were more uniformly distributed throughout the animal. It is important to note that PEGylation tends to increase the diameter of the NPs by about 10 nm, similar to other conjugation methods. However, an essential aspect of PEGylation is the characterization of NPs, and the dynamic light scattering (DLS) is a crucial technique that can provide three critical parameters: size; zeta potential that measures the surface charge of the NPs and determines their colloidal stability (values between −10 and +10 mV are neutral, while values greater than +30 mV or less than −30 mV are considered strongly cationic and strongly anionic, respectively), and size distribution [15]. The selection of ligands to bind the PEGylated-NPs may vary depending on the application. The purpose of having PEGylated-NPs with ligands is to target specific receptors on the surface of cancer cells and to allow for retention in the area due to the enhanced permeability and retention effect (EPR). A variety of ligands can be used, including molecules, peptides, proteins, antibodies, aptamers, among others [12, 16–19].

However, PEG may undergo degradation due to light, stress, or heat. Some authors have addressed this issue by combining PEG with copolymers such as PVP and poly(lactic-co-glycolic acid) or PLGA [20]. With these challenges, research with PEG continues to be relevant, as it has proven to be an important tool for achieving the biocompatibility of NPs.

2.2 Polyethyleneimine

Polyethyleneimine is a very versatile aliphatic polymer that contains primary, secondary, and tertiary amino groups, with a ratio of 1:2:1 [21]. It has found numerous applications in non-viral gene delivery and therapy for *in vitro* and *in vivo* models. In addition, PEI has been used for non-pharmaceutical applications, such as water purification and shampoo manufacturing. For instance, Ge and collaborators [22] developed near-infrared emitting nanoparticles coated with PEI and gold nanorods coated with dithiothreitol to detect arsenic (III), while Pan et al. [23] synthesized PEI-coated upconversion nanoparticles for use as an optical probe to determine the water content in organic solvents.

Polyethyleneimine-modified nanoparticles have also been explored for various biomedical applications. Mi et al. [24] developed luminescent NPs coated with PEI that can bind to antibodies through their amino groups, resulting in tunable colors. Xu et al. [25] functionalized NPs with folic acid and polycaprolactone/PEI for *in vivo* drug delivery in SKOV-3 cancer cells. Their results showed that their method was more effective in killing cancer cells than free doxorubicin. PEI-NPs have also been used for pulmonary gene delivery. Bivas-Benita et al. [26] developed a PLGA-PEI-NP that can deliver genes to the lung epithelium using Calu-3 cells. Huh et al. [27] used PEI-NPs composed with glycol chitosan and encapsulated with siRNA, which significantly inhibited red fluorescent protein (RFP) gene expression in B16-F10-bearing mice cells.

PEI nanoparticles represent an important tool especially for drug delivery of anticancer drugs and also gene therapy applications, among others.

2.3 Polyvinylpyrrolidone

PVP is commonly used as a coating for silver NPs and as a drug carrier [25, 28, 29]. However, several authors have also used PVP as a coating for UCNPs [25, 30–34].

PVP is a versatile coating because it can work as a NP dispersant or as a surface stabilizer, and it also has reducing properties. Its functional groups, which include C=O, C–N, and CH_2, enable it to control the growth of certain aspects by binding onto others, providing biocompatibility to the NPs [29, 35].

Johnson et al. [34] synthesized β-$NaYF_4$:Yb^{3+}/Er^{3+} UCNPs and used PVP to replace the oleate surface ligands. This modification makes the UCNPs water-dispersible, which is crucial for *in vivo* applications. Additionally, PVP is biocompatible, has a prolonged blood circulation time, and shows low accumulation in vital organs. Zou et al. [33] prepared UCNP $NaYF_4$:Yb^{3+}/Er^{3+} embedded into PVP nanotubes using the electrospinning method, resulting in an intense emission of the UCNPs compared to bare UCNPs. Due to their biocompatibility, these modified NPs may have important applications in biomedicine.

2.4 Polyacrylic acid

PAA is a hydrophilic and pH-responsive polymer that can replace hydrophobic ligands on the surface of NPs, making it an excellent candidate for *in vivo* and *in vitro* applications [36]. Its biocompatibility and other desirable qualities make it an attractive coating option for various types of NPs [37–41].

Hilderbrand et al. [42] synthesized UCNPs coated with PAA and linked amino-PEG to the carboxyl groups of the PAA. The resulting modified UCNPs were non-cytotoxic and displayed good NIR emission. Wang et al. [41] also prepared UCNPs YF_3:Yb^{3+}/Er^{3+} with NIR emission and coated with PAA, resulting in strong luminescence. In a study by Xiong et al. [40], PAA-coated UCNPs were shown to have excellent biodistribution and cellular uptake in mice, with no observed toxicity, suggesting that these NPs could be used for long-term therapy and bioimaging studies *in vivo*. Additionally, Jia et al. [36] investigated the effects of doxorubicin hydrochloride (DOX) and PAA-coated UCNPs (DOX@PAA-UCNPs) on HeLa cells and found that the UCNPs were biocompatible and effective as a drug carrier.

In summary, PAA is a very versatile polymer that can be used to coat on various types of NPs for a wide range of biomedical applications.

2.5 Silica

Silica (SiO_2) is a commonly used coating material for various types of NPs due to its favorable properties, including biocompatibility, thermodynamic stability, low toxicity, colloidal stability, ease synthesis, and scalability. Two main methods are generally used for producing the coating: sol-gel in a reverse micelle nanoreactor and the Stöber method [43, 44]. However, achieving a complete and homogeneous coating is a significant challenge, and Ureña-Horno et al. [45] developed a method for coating UCNPs with silica. By determining the optimal concentration of nanoparticles, they were able to achieve high yields of homogeneous functionalization and prevent agglomeration.

Hlaváček et al. [46] employed agarose gel electrophoresis for the purification of silica-coated UCNPs and for the separation of the protein-UCNPs from surplus reagents. This work represents a significant advancement in nanoparticle separation and measurement of their size and surface charge. In another study, also, Gnanasammandhan et al. [47] used silica-coated UCNPs for photoactivation in two specific applications: photodynamic therapy (PpDt) and photoactivated control of gene expression. The UCNPs were coated with PEG and functionalized with FA to target specific tumors, and their protocols for photoactivation therapy are valuable for future studies.

Overall, the efficient coating and functionalization of nanoparticles with silica are vital for their successful use in various applications, and these studies provide important insights and protocols for achieving these goals.

3. Toxicology of nanoparticles

The study of the toxicological effects elicited by NPs on cells and organisms is crucial in biomedical-nanotechnology applications. Thus, it is important to ensure that NPs are not cytotoxic or genotoxic. **Table 1** summarizes various approaches used by different authors for the toxicological assessment of nanoparticles.

3.1 Cytotoxicity assays

Assessing the cytotoxicity of new agents or nanomaterials is a crucial step in evaluating their potential biomedical applications. *In vitro* cell culture tests are preferred over *in vivo* animals test for ethical, speed, and cost reasons. However, cell cultures tend to be susceptible to various environmental factors, such as pH, nutrients, and temperature, which may interfere with the interpretation of the results. Therefore, it is important to ensure that the observed cell viability is observed solely due to the toxicity of the nanomaterials being tested, rather than environmental factors. Performing a range of tests with different concentrations of NPs and consistent experimental conditions enhances the validity of results [56, 57].

The MTT assay, based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide by dehydrogenase enzymes, is one of the most common methods to assess cell viability, as it measures mitochondrial activity in living cells [58–60]. This assay detects living cells, and the results are easily read using a multiwell scanning spectrophotometer (ELISA plate reader). Several authors have successfully used this assay, including those listed in **Table 1** [48, 50, 55].

Another variation of the MTT assay is the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, which uses MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], and phenazine ethosulfate, instead of MTT. Bahadar et al. [61] used both methods to evaluate the cytotoxicity of different metallic and non-metallic NPs on cells.

Other methods for measuring cell viability include the trypan blue and neutral red assays, which detect dead cells based on dye penetration into cell membrane. Ramírez-García et al. [62] used the trypan blue assay to measure the cell viability of zinc-gallium luminescent NPs; also, Zairov et al. [63] used gadolinium-based luminescent NPs with PC12 cells for obtaining low cytotoxicity, and the viability of the living cells was measured with a hemocytometer.

Live/dead viability assay, which measures the number of damaged cells, uses calcein acetoxymethyl (calcein AM) and ethidium homodimer. This method was mostly used to test the cytotoxicity exerted by gold nanoshells, silver, silica NPs, or fullerenes on cells [64]. The water-soluble tetrazolium (WST-1) assay is another method that measures mitochondrial activity by transforming the light-red tetrazolium salt into dark-red formazan salt due to the mitochondrial activity in living cells. Braun et al. [65] evaluated silica NPs with C2C12 cells using MTT and WST assays, and described that the MTT assay overestimated the low and medium cytotoxicity of the NPs, while the WST assay underestimates the particle concentrations studied.

NPs	Cytotoxicity assay	ROS quantification	Genotoxicity/gene expression	Stability or distribution	Cells/organism	References
Y_2O_3:Eu and Yb/Er	MTT assay	Comet assay	Comet assay	—	HeLa, MCF-7 cells	[48, 49]
$NaYF_4$:Yb/Er	MTT assay	—	—	—	RAW 264.7 cells	[50]
$NaYF_4$: Yb/Er	—	Oxidative stress assay	—	Stability assay/ biodistribution studied	Fetal bovine serum/ mice	[51, 52]
NPs-PEI-SiRNA	RFP	—	RFP expressing B16F10 cells	—	Murine melanoma/RFP/ B16-F10 cells	[27]
Yb_2O_3:Gd	A. cepa chromosomal aberration assay method	—	A. Cepa genotoxic studies	-	*E. coli* and *S. aureus*	[53]
Gd_2O_2: Yb/Tm	*In vitro* biodegradation assay	—	—	Biodistribution and toxicity in organs studied	Mice *in vivo*/blood *in vitro*	[54]
Y_2O_3	MTT assay	—	—	—	Human breast cancer	[55]

Table 1.
Toxicological assessment of NPs described by different authors.

There are alternative approaches to assess the cytotoxicity of NPs: For instance, Das et al. [66] carried out a study on the toxic effects of three types of functionalized UCNPs: oleate ligands-NPs, PEG-NPs, and bilayer PEG-oleate-NPs. They employed the calcein and propidium iodide viability assay and concluded that the bilayer NPs exhibited significant toxicity due to functionalization. In another study, Malvindi et al. [67] evaluated the cytotoxicity of silica-coated iron oxide NPs using the WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay and lactate dehydrogenase release (LDH assay) to analyze cell viability and cell membrane integrity. The NPs demonstrated good internalization in HeLa cells with no observed toxicity. Meindl et al. [68], on the other hand, assessed the cytotoxicity of NPs by measuring intracellular calcium levels, providing an example of an alternative approach for toxicity evaluation. It is important to select an appropriate method for assessing cytotoxicity, with suitable experimental parameters and consistent concentrations of NPs and exposition times across different studies. Otherwise, non-toxic NPs may yield misleading results due to factors such as cellular senescence.

3.2 Reactive oxygen species/reactive nitrogen species

The production of reactive nitrogen species (RNS), such as nitric oxide (NO), is closely associated with inflammatory responses and can react with oxygen to produce ROS. When NPs interact with cells, they may induce cell death by triggering the production of NO. The production of RNS is regulated by the enzyme nitric oxide synthase (NOS), while ROS production is regulated by NAD(P)H oxidase isoforms. Excessive ROS production can cause oxidative stress, leading to damage in the cell membrane, proteins, lipids, or DNA. However, low or moderate concentrations of ROS/RNS are beneficial, as they can help to defend against infections [69–71].

Several studies have demonstrated that metal and silica nanoparticles can induce oxidative stress and inflammation. The reactivity at the target sites and the surface area are two crucial factors affecting these outcomes. In a study conducted by Tran et al. [72], the effects of nanoparticles' surface area on lung health were investigated. They demonstrated that NPs with a higher surface area tend to be retained and accumulate in the lungs, reaching a saturation point where they become less susceptible to phagocytosis and exhibit reduced mobility. This overload effect stimulates macrophages, leading to the production of inflammatory responses, including tumor necrosis factor.

In a recent study, Wang [73] investigated the use of ROS probes to detect and visualize ROS production in living cells. The most commonly used ROS include H_2O_2, $^1O_2$, $O_2^{\bullet -}$, ClO−, ONOO−, and •OH. Luminescent NPs were found to be effective probes for detecting H_2O_2 and other ROS forms in living cell systems. The authors suggest that these nanoprobes may have promising therapeutic applications for sensing ROS.

3.3 Genotoxicity

When conducting deeper cytotoxicity studies, determining the genotoxic potential of NPs is often necessary. Various authors have employed different methods to ensure single- and double-stranded DNA breakage caused by NPs exposure. One of the most used methods is the flow cytometry that differentiates among various cell populations, between cell size, and complexity (granularity) through a laser beam [74]. Intercalating dyes such as propidium iodide can be used to measure DNA