Chapter 9
Cellular Mechanisms in Nanomaterial Internalization, Intracellular Trafficking, and Toxicity

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Abstract Nanomaterials are expected to have a significant impact on medicine, although they still need to overcome several challenges before they are widely used. Understanding the molecular interaction of nanomaterials in the context of the cellular environment is crucial for the success of nanomaterials. Therefore, mechanisms responsible for nanomaterial internalization have attracted great attention in the scientific community. These mechanisms greatly impact intracellular trafficking and cellular processing of nanomaterials. Here we discuss the major endocytic pathways by which nanomaterials can be internalized by cells, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis. In addition, intracellular routing, metabolism of nanomaterials, and undesirable effects of nanotoxicology are discussed. Finally, the role of in vitro studies to evaluate the potential toxic effects of nanomaterials was critically analyzed.

9.1 Introduction

During this last century scientists have revealed many of the most fundamental mysteries about life at the molecular level. This knowledge has given us the power to manipulate and control matter at the nanoscale. This understanding has attracted growing attention in industry and academia, thereby fueling outstanding progress in this field. The design, synthesis, and application of materials at a nanoscale (1–1,000 nm) is called *nanotechnology,* and the nanomaterials created can exhibit very interesting properties (Buzea et al. 2007; McNeil 2005). Given their size,

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nanomaterials often exhibit different physical (mechanical, electrical, optical, etc.) properties when compared to macroscopic systems. Consequently, these changes in physical properties of nanomaterials are reflected in their chemical properties. The increase in surface area to volume ratio modifies mechanical properties, which in turn make nanomaterials much more reactive than their bulkier material counterparts (McNeil 2005). These novel properties afford nanomaterials' unique applications, enabling them to substantially improve the effectiveness of a number of existing products that could result in a substantial impact on science and technology in the twenty-first century (Yan et al. 2012). Thus, nanotechnology has the potential to introduce dramatic improvements in the human quality of life.

Nanotechnology's ability to manipulate matter provides a vast range of applications, from quantum computers to self-cleaning clothes. Among them, the branch that has been attracting enormous attention from scientists is *nanomedicine*; defined as the medical application of nanotechnology, it allows monitoring, repairing, controlling, and even constructing biological systems at the molecular scale (El-Ansary and Al-Daihan 2009). Nanomedicine is an interdisciplinary field that involves biology, chemistry, physics, medicine, material science, and biomedical engineering and has proven to be a very fertile research field. One of the main reasons for this high productivity is that nanomedicine can offer therapeutic approaches and disease diagnostics that cannot be achieved by conventional strategies (Cabral et al. 2011; El-Ansary and Al-Daihan 2009; Rajendran et al. 2010; Yan et al. 2012). For example, nanomaterials can be used as diagnostic devices identifying diseases at earlier stages, hence increasing the effectiveness of the treatment and decreasing its cost. In addition, some particles have shown therapeutic properties, which opens new possibility for their applications.

Different nanomaterials have been applied in biotechnology, including polymeric nanoparticles, quantum dots, liposomes, polymer-drug conjugates, dendrimers, lipid nanoparticles, silica nanoparticles, carbon fullerenes, nanotubes (single and multi-walled), metal oxides (titanium dioxide, zinc oxide, cerium oxide, iron oxide), and nanoscale metals (silver, gold, copper), among others. Irrespective of their applications, materials at the nanoscale can enter the human body and this may occur via different routes: intravenous, dermal, subcutaneous, inhalation, intraperitoneal, and oral. In this report, we are particularly focused on how this new technology interacts with living things at the cellular level. Due to their size, nanomaterials have greater potential to travel inside living organisms than other materials or larger particles (McNeil 2005). Eventually, these nanomaterials can interact with cells at the plasma membrane and lead to their internalization through the process of *endocytosis*.

Endocytosis is a biological process highly conserved across species and cell types through which cells internalize nutrients, regulate signal transduction, and modulate plasma membrane composition. Endocytosis begins with plasma membrane invagination to bring in the cargo. This is followed by membrane budding, whereby specific proteins (e.g., dynamin) are pinched off so that the cargo can undergo subsequent internalization via the endocytic pathway (Aguilar and Wendland 2005). Inside the cells these vesicles are called endosomes.

Endosomes are decorated with a large variety of proteins on their surface membrane, which has a different composition from the inside surface. Similar to other cargos, nanomaterials can be delivered to different cellular and extracellular destinations. Nanomaterials can reach degradative compartments (e.g., late endosomes or lysosomes), be recycled back to the extracellular milieu, be transported across cells (i.e., transcytosis), or reach different organelles (e.g., Golgi apparatus, mitochondria) (Sahay et al. 2010a).

Nevertheless, once inside the cell, the physicochemical characteristics that make nanomaterials so useful can also be the main reason they might be dangerous to cells, and at a higher level to human health. The high reactivity of nanomaterials can lead to toxicity via mechanisms that include induction of oxidative stress, inflammation, organelle dysfunction, and change of cellular morphology. With regard to size, the upper limit of any nanomaterial that can undergo internalization by nonprofessional phagocytic cells was thought to be about 150 nm. However, Gratton and coworkers showed that this upper-size limit needs to be reconsidered, since they demonstrated the internalization of nanoparticles of up to 3 μm by nonprofessional phagocytic cells (Gratton et al. 2008). Therefore, nanotoxicology should not be limited to nanomaterials in the range of 1–100 nm, since microparticles of up to 3 μm can also be internalized via endocytosis (Gratton et al. 2008), and theoretically, once inside the cell, their potential for causing harm is high (Zhao et al. 2011). To avoid these problems, nanomaterials must be engineered from either materials that are biocompatible, nontoxic, and biodegradable or those materials that have minimal toxic effects (Ai et al. 2011).

There has been growing concern with the potential health risks posed by nanoscale materials, and thus, a subdiscipline in this field has emerged, namely, *nanotoxicology* (Donaldson et al. 2004). Nanotoxicology is the study of the toxicity of nanomaterials. Nanotoxicology also deals with understanding the interactions between nanostructures and biological systems, that is, trying to elucidate the relationship between the physicochemical properties (e.g., size, shape, surface chemistry, composition, and aggregation) of nanomaterials and the toxic effects elicited at the cellular level (Oberdörster et al. 2005). The increase in the number of publications related to nanotechnology has drawn the attention of Federal agencies, which underscores the increased importance given to nanotoxicology. Furthermore, some studies have found that publications lack a critical review of the nanotoxicity of new nanomaterials, thereby presenting false-positive results. Thus, agencies have been conducting research that is expected to provide more reliable risk evaluations (Ai et al. 2011; Donaldson et al. 2004; Maynard et al. 2011; Oberdörster et al. 2005).

Although there is concern about the toxicity of new nanomaterials, not all of this can be evaluated in vivo. It has been estimated that a billion dollars and about 30–50 years are needed to conduct traditional in vivo studies on the nanomaterials currently under commercialization (Walker and Bucher 2009). As an alternative, there is a huge campaign in the scientific community to evaluate the potential toxic effects of nanomaterials in vitro. This would contribute not only to a faster development of this field but also to our knowledge about nanomaterial interactions

at the molecular and cellular level, which could help us understand their interactions with living things and their environmental impact. Ultimately, the development of nanomaterials and their future commercial applications will be a challenge not only for companies but also for state regulatory agencies that must guarantee their safety for the work force, consumers, and the environment.

9.2 Cellular Endocytosis as a Mechanism of Nanomaterial Internalization

The plasma membrane is selectively permeable, determines the cellular boundary, is not static but dynamic, and has evolved several mechanisms to control the communication between the cytosol and extracellular environment. For example, oxygen, carbon dioxide, and small hydrophobic or nonpolar molecules are able to cross freely across the plasma membrane. Similarly, many small polar molecules, such as ions and amino acids, can get through the plasma membrane via active transport mediated by integral membrane protein pumps or ion channels. In contrast, the plasma membrane is highly impermeable to bigger and polar structures, such as nanomaterials. A mechanism that permits nanomaterial cellular internalization is endocytosis (Doherty and Mcmahon 2009). This mechanism is involved in many normal physiological processes, such as the uptake of extracellular nutrients, regulation of cell surface receptor levels, maintenance of cholesterol homeostasis, and antigen presentation. Moreover, many diseases and pathogenic conditions, including atherosclerosis and diabetes, are the result of abnormalities in endocytic processes. Even pathogens, including viruses, symbiotic microorganisms, and toxins, exploit endocytic pathways to gain entry into the cell. In addition, this ATP-dependent and well-coordinated cellular process, can mediate internalization of several kinds of nanomaterials. It starts with the invagination of the plasma membrane and leads to the internalization of the nanomaterial within an endocytic vesicle in the cytoplasm (Canton and Battaglia 2012; Sahay et al. 2010a).

Endocytosis, performed by professional phagocytes, such as macrophages, neutrophils, monocytes, and dendritic cells, is defined as phagocytosis, whereas pinocytosis is performed by virtually all other eukaryotic cell types (Doherty and Mcmahon 2009; Zhao et al. 2011). Pinocytosis can occur via different morphological and biochemical mechanisms, such as clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), macropinocytosis, and clathrin- and caveolae-independent endocytosis. The new pathways that are subclassified as clathrin and caveolae independent include Arf6-dependent, flotillin-dependent, Cdc42-dependent, and RhoA-dependent endocytosis (Canton and Battaglia 2012; Sahay et al. 2010a).

The study of the specific endocytic pathway that is used by nanomaterials is mechanistically important because it determines the intracellular fate of these nanomaterials, and thus is essential for determining their efficiency and possible

toxicity. It is well established that this is a cell type-, material composition-, and concentration-dependent process (Hillaireau and Couvreur 2009; Zhao et al. 2011). Here we discuss the major endocytic pathways: CME, caveolin-mediated endocytosis, and macropinocytosis.

9.2.1 Clathrin-Mediated Endocytosis

CME is a key process for carrying out many fundamental physiologic functions in eukaryotic cells. This mechanism is involved in many cellular processes, including the uptake of nutrients, such as transferrin and riboflavin (Bareford et al. 2008; Gao et al. 2005), cholesterol incorporation into cells by low-density lipoprotein (LDL), and downregulation of cell signaling by internalization and degradation of receptors. The involvement of CME in several processes has made this a central topic of study in different fields, and therefore, CME is the best-understood endocytic pathway (González-Gaitán and Stenmark 2003; Hillaireau and Couvreur 2009). Furthermore, viruses seem to take particular advantage of this pathway to gain entry into host cells (Canton and Battaglia 2012; Mudhakir and Harashima 2009).

Clathrin forms a triskelion by combining three clathrin heavy chains and three light chains. This structure is associated with numerous adaptor proteins that help deform the cytoplasmic face of the plasma membrane, forming pits that are pinched off by the protein dynamin (Edeling et al. 2006; Hinrichsen et al. 2006; Marsh and McMahon 1999; Ungewickell and Hinrichsen 2007). Dynamin is a large GTPase responsible for membrane scission, and it is assisted by actin and myosin motor proteins. As the process continues, clathrin-coated vesicles (CCV) are formed in the cytoplasm after the clathrin triskelion helps form a mechanical scaffold on the vesicle surface, then the units are released and recycled back to form new vesicles. This process is very dynamic, and it is estimated that cultured cells take about 1 min to assemble CCV, and hundreds and up to a thousand CCV can be formed every minute (Marsh and McMahon 1999).

CME is particularly important for nanomaterial internalization, since nanomaterials primarily end up in lysosomes. Although some nanocarriers can use this as a mechanism to trigger the release of their contents, most cargo is completely degraded in the lysosomal compartment, a process especially critical for disposal of discarded genetic material. Researchers researchers are trying to outline generalizations about nanomaterial physicochemical properties and the type of endocytic pathway that mediates their processing, and for some structures it has been possible to draw some conclusions. This seems to be the case for lipoplexes, complexes formed by nucleic acids and liposomes. Several studies have suggested that these nanocarriers enter cells via CME (Rejman et al. 2005; Zuhorn et al. 2002). Similarly, different types of nanoparticles seem to be internalized by CME, such as solid lipid nanoparticles (Martins et al. 2012), polysaccharide cationic nanoparticles (Dombu et al. 2010), PLGA-based nanoparticles (Benfer and Kissel 2012), diamond nanoparticles (Faklaris et al. 2009), and silver

nanoparticles (Greulich et al. 2011). Furthermore, professional phagocytes, such as macrophages, can use CME to internalize silver nanoparticles (Kim and Choi 2012).

Several inhibitors and inhibitory mechanisms have been used to study and characterize this endocytic process, such as hypertonic sucrose, K^+ depletion, and chlorpromazine (Heuser and Anderson 1989; Madshus et al. 1987; Wang et al. 1993). In addition, the inhibition of actin polymerization with latrunculin A and cytochalasin D, which compromises the internalization via CME, has also been investigated (Sahay et al. 2010a). Furthermore, macromolecules that are normally internalized via CME can be used as endocytic markers to study this process further. The most often used markers are the iron transport protein transferrin and LDL (Sahay et al. 2010a).

9.2.2 Caveolae-Mediated Endocytosis

Caveolae are flask-shaped invaginations of the plasma membrane, which are found in domains enriched with cholesterol and sphingolipids (Bastiani and Parton 2010; Lajoie and Nabi 2007; Nichols 2003). CvME is involved in several biological processes, including transcytosis, cell signaling, lipid regulation, and also many diseases, such as cancer, diabetes, and viral infections (Hayer et al. 2010). CvME is abundant in some cell types, such as muscle cells, endothelial cells, fibroblasts, and adipocytes, and rare in others, such as neurons and leukocytes (Bastiani and Parton 2010; Doherty and Mcmahon 2009).

The conclusive characteristic of caveolae is the presence of caveolin (Cav) proteins. In mammalian cells, the caveolin gene family is comprised of three members: caveolin-1 (Cav-1), caveolin-2 (Cav-2), and caveolin-3 (Cav-3). Cav-1 and Cav-2 are widely found in different body tissues, while Cav-3 seems to be found only in muscles cells. Cav-1 is anchored to the plasma membrane in domains rich in cholesterol, sphingomyelin, saturated fatty acids, and glycosphingolipids (e.g., GM1). These domains can also include glycosylphosphatidylinositol (GPI)-linked proteins (Doherty and Mcmahon 2009; Parton and Simons 2007). Although Cav-1 is necessary for biogenesis of caveolae (Doherty and Mcmahon 2009), its ability to induce membrane curvature is currently under debate. It has been suggested that new cavin proteins form a complex that can stabilize the membrane curvature to produce the classic flask shape of caveolae, therefore regulating caveola structure and function (Hill et al. 2008). In addition, CvME also relies on specific adaptor proteins to mediate this process, and similar to CME, it relies on dynamin to pinch off the budding vesicle. Furthermore, some viruses can also exploit CvME to gain entry into cells, such as Simian virus 40, Shiga toxin, and the cholera toxin B subunit; the latter is used as a marker for CvME. CvME is also responsible for the uptake of nutrients, such as folic acid, albumin, and cholesterol (Hillaireau and Couvreur 2009).

For many years it was thought that the CvME pathway could bypass lysosomal enzymatic degradation. For this reason, this pathway was believed to be beneficial for cellular delivery of drugs sensitive to lysosomal degradation, especially the delivery of peptides, proteins, and nucleic acids among others. Hypothetically, cargo internalized by CvME would lead to caveosomes, special endosomal compartments with neutral pH (Khalil et al. 2006; Pelkmans and Helenius 2002; Pelkmans et al. 2001; Sahay et al. 2010a). Recently, the same authors who described this structure have reported that caveosomes are actually an artifact present in cells overexpressing caveolin (Hayer et al. 2010; Parton and Howes 2010). These findings call for a careful reassessment of the data already published regarding intracellular localization and degradation of nanomaterials. Nonetheless, some researchers found that CvME does avoid or delay lysosomal degradation. For example, highly compacted DNA nanoparticles internalized by CvME exhibited negligible colocalization with LysoTracker, a lysosomal marker, for up to 4-h post-incubation in human bronchial epithelial (BEAS-2B) (Kim et al. 2012). As discussed before, avoiding the lysosomal compartment can be beneficial for the delivery of nucleic acids. Del Pozo-Rodríguez and coworkers observed that solid lipid nanoparticles decorated with proline-rich peptides shifted their internalization pathway from CME to CvME, resulting in higher transfection efficiency in both HEK293 and ARPE-19 cells (del Pozo-Rodríguez et al. 2009). However, recent findings have demonstrated that the CvME pathway can direct nanomaterials to lysosomes (Ekkapongpisit et al. 2012; Sahay et al. 2010b). Given these studies, it is apparent that more work is required to understand nanomaterial trafficking trough CvME and especially the functions associated with caveolin and the fate of nanomaterials in caveolar trafficking (Parton and Howes 2010).

9.2.3 Macropinocytosis

Macropinocytosis was the first endocytic pathway described, although its biological roles have only recently been explored. Macropinocytosis seems to be important for cell motility; therefore, it was studied in the context of cancer progression and metastasis (Lim and Gleeson 2011). It is also important for some specialized cell types, such as professional phagocytes, where it plays significant roles in immune system processes, such as antigen presentation and clearance of apoptotic bodies and necrotic cells. It is a constitutive process in professional phagocytes, but in nonprofessional phagocytes, it has to be induced in response to growth factors, such as epidermal growth factor (Jones 2007; Mercer and Helenius 2009). Interestingly, macropinocytosis can also be induced by bacteria and viruses; thus, these organisms can take advantage of this endocytic pathway to enter host cells. Other pathogens can also enter host cells via macropinocytosis, such as protozoa and prions (Barrias et al. 2012; Kerr and Teasdale 2009; Lim and Gleeson 2011; Mercer and Helenius 2009).

Macropinocytosis can be defined as the bulk uptake of large amounts of extracellular material. It is characterized by the ruffling of the plasma membrane and is induced by global activation of the actin cytoskeleton. In this manner, the entire cell surface starts ruffling or blebbing, and these protrusions fold back and fuse with the plasma membrane. This fusion results in large endocytic vacuoles, called macropinosomes that are heterogeneous in size and morphology (Falcone et al. 2006; Jones 2007). Macropinosomes are easy to distinguish from other vesicles formed during pinocytosis, since they are substantially larger (0.5–10 μm), non-coated structures that shrink during their intracellular maturation. For some time, it was believed that macropinosome formation was a dynamin-independent process, but in some cells this does not seem to be the case (Orth and McNiven 2006). Furthermore, a macropinocytosis-like pathway that is dynamin dependent was recently described for the internalization of quantum dots (Iversen et al. 2012).

Although the definition of macropinocytosis gives the impression of a random process, it is actually a very complex and well-coordinated cellular event (Falcone et al. 2006). External stimuli usually initiate the process through the activation of receptor tyrosine kinases (RTKs), which in turn activate key signaling proteins, such as Ras, PLC, and PI 3-kinase. These proteins coordinate the dynamic changes in actin filaments and generate plasma membrane ruffling (Mercer and Helenius 2009; Swanson and Watts 1995). Additionally, Rho family members and their upstream effectors are also required for the actin polymerization machinery and ruffling (Jones 2007).

Given their nature, macropinosomes can be identified through the use of fluid phase markers, such as dextrans, horseradish peroxidase, and lucifer yellow. Inhibitors can also be used to help examine this process, such as cytochalasin D, which inhibits actin polymerization, and amiloride; however, its underlying mechanism remains unknown (Falcone et al. 2006; Kerr and Teasdale 2009; Lim and Gleeson 2011; Swanson and Watts 1995).

In addition to internalizing viruses, bacteria, and others pathogens, macropinocytosis can also be used to internalize nanomaterials. Macropinocytosis has been suggested as an effective endocytic pathway for drug delivery into cells. Many arginine-rich cell-penetrating peptides such as octo-arginine and human immunodeficiency virus transactivator of transcription (TAT) peptides exploit the process of macropinocytosis to effectively deliver peptides and proteins into cells (Kaplan et al. 2005; Nishimura et al. 2008). Mesoporous silica nanoparticle rods were more effective in the delivery of chemotherapeutic agents in HeLa cells when endocytosed via macropinocytosis (Meng et al. 2011). Particle size is an important determinant for internalization by macropinocytosis, as demonstrated by Vollrath and coworkers, who used using nanoparticles based on poly-(methyl methacrylate) in Hela cells. These authors found that CME is the predominant pathway for internalization of smaller nanoparticles ($<$200 nm), whereas macropinocytosis is the main pathway for internalization of larger particles ($>$300 nm) (Vollrath et al. 2012). Nevertheless, small particles can also be internalized by macropinocytosis. Iversen and coworkers showed that even small

particles (30 nm), i.e., ricin-coupled quantum dot nanoparticles, are internalized by Hela cells via macropinocytosis (Iversen et al. 2012).

9.2.4 Additional Internalization Pathways for Nanomaterials

Recently, the study of nanomaterial internalization has attracted great attention (Canton and Battaglia 2012; Doherty and Mcmahon 2009; Hansen and Nichols 2009; Hillaireau and Couvreur 2009; Sahay et al. 2010a; Scita and Di Fiore 2010; Verma and Stellacci 2010; Zaki and Tirelli 2010; Zhao et al. 2011). It is important to determine not only the effectiveness of a new nanomaterial but also its cytotoxicity. One must be careful to interpret data before deciding the specific endocytic pathway involved. In addition to CME, CvME, and macropinocytosis, other new endocytic pathways have been described, such as RhoA-, CDC42-, and flotillin-mediated endocytosis. These pathways have already been described for some nanomaterials, and it is expected more pathways will be described (Kasper et al. 2013). Some particles were described as not following the classical pathways (clathrin, caveolin, and macropinocytosis). For example, carboxyl-modified fluorescent polystyrene nanoparticles were internalized by HeLa cells via a nonclassical pathway (Lai et al. 2007). These particles may have taken one of the recently described pathways or a new and yet undescribed pathway. Actually, cells can use more than one endocytic pathway to internalize nanomaterials. For example, dendrimers were internalized via both CvME and macropinocytosis, and in the same paper, the authors described a different formulation of dendrimers that were internalized via CvME, CME, and macropinocytosis (Saovapakhiran et al. 2009). In addition, new mechanisms of nanocarrier-cell surface interactions have been described, such as actin-rich filopodial extensions that are responsible for a majority of the internalization of lipids and polymers carrying DNA into Hela cells (Rehman et al. 2012).

Endocytic markers and inhibitors can be very useful in determining the endocytic internalization pathway of new nanomaterials, however data obtained using these methods should be interpreted with caution. Although the cholera toxin B subunit has been widely used as a marker for CvME, it can also be endocytosed by several pathways, including CME (Torgersen et al. 2001). Furthermore, because the fluorescently labeled form of the toxin can bind to numerous nanomaterials even before being endocytosed, it can lead to false-positive colocalization. Endocytic inhibitors can also present problems, such as toxic effects, and they can be nonspecific. For instance, methyl-β-cyclodextrin is commonly used to remove cholesterol from the plasma membrane and disturb CvME, however it can also disturb CME, depending on the concentration used and cell type (Rodal et al. 1999; Subtil et al. 1999). These examples are not intended to exhaustively cover the subject, but to highlight the complexity behind the mechanisms used by cells to internalize nanomaterials. They also suggest that care should be taken in performing experiments and analyzing results to properly determine the specific

endocytic pathway used for internalization of nanomaterials by specific cell types. The reader is referred to these articles, which describe specific pitfalls and limitations of techniques for determining the endocytic pathways used by nanomaterials (Vercauteren et al. 2010; Zaki and Tirelli 2010).

New techniques have been developed to study endocytosis and intracellular trafficking of nanomaterials. Recently a novel, highly sensitive, and quantitative technique was developed to elucidate the precise nature of the membrane compartments through which nanomaterials are routed following internalization into cells. The authors used a rapid, multicolor, live-cell, confocal fluorescence microscopy approach that captures images in three dimensions and thereby provides details about the intracellular interactions between nanoparticles and the intracellular components (Sandin et al. 2012). These techniques can add new insight to the rare and fast events by which nanomaterials interact with the intracellular environment.

9.3 Intracellular Trafficking of Nanomaterials

After examining the internalization pathways for nanomaterials, the next areas to be addressed are the intracellular trafficking and localization of nanomaterials. These areas are critical for optimization and characterization of nanomaterials aimed for intracellular-targeted drug delivery. Currently, the impact of nanotechnology on medicine is already significant, since many nanomaterials are approved for clinical use. However, most nanomaterials can only improve the therapeutic index of drugs by reducing their toxicity or enhancing their efficacy. For decades to come, it is expected that nanotechnology will make an enormous impact on medicine and human healthcare; however to achieve this, the next generation of nanomaterials will have to improve the ability to reach specific tissues, cells, and intracellular targets. These advances would improve the nanocarriers efficacy or reduce their toxicity. This need can explain the great attention that has been given to the rational design of nanomaterials and strategies to properly target them to subcellular compartments (Bareford and Swaan 2007; Chou et al. 2010; Murakami et al. 2011; Petros and DeSimone 2010; Prokop and Davidson 2008; Rajendran et al. 2010; Torchilin 2006).

Regardless of the internalization pathway used by cells for taking up nanomaterials, the first compartment that receives the cargo is the early endosome (EE). Currently, EEs are recognized as the main sorting station in the endocytic pathway (Huotari and Helenius 2011). In fact, one of the first steps to confirm that nanomaterials have entered cells through endocytosis is to demonstrate that they are in the EE. The EE is characterized by the presence of the early endosomal antigen-1 (EEA1) protein, the most widely used marker for EE, or Rab5, a protein member of the Ras superfamily of small Rab GTPases (Canton and Battaglia 2012; Scita and Di Fiore 2010). Subsequently, nanomaterials follow the classical intracellular trafficking pathway, meaning that they can be recycled back to the plasma

membrane, delivered to the trans-Golgi network or across the cell, or undergo degradation in the lysosomes (Huotari and Helenius 2011; Jovic et al. 2010). Here we will discuss some examples of intracellular trafficking and their consequences. Lysosomal degradation and the recycling of nanomaterials to the plasma membrane are discussed in more detail in the next section.

Among the different fates that nanoparticles can face inside the cell, avoiding lysosomal degradation is the most important for achieving an effective therapeutic effect. Ming and coworkers demonstrated that an antisense oligonucleotide conjugated with a bombesin peptide bypassed lysosomal degradation and accumulated in the trans-Golgi network, representing an efficient system for intracellular delivery of oligonucleotides (Ming et al. 2010). In agreement with these findings, Chang and coworkers showed an accumulation of gold nanoparticles within the endoplasmic reticulum and Golgi apparatus of B16F10 melanoma cells. The authors found that combining gold nanoparticle treatment with radiotherapy resulted in an increase in the apoptotic potential of the therapy, suggesting that gold nanoparticles can improve the clinical outcome of melanoma radiotherapy (Chang et al. 2008). Fichter compared the efficiency of two gene delivery systems, glycofect and linear polyethylenimine polymer, in ER H9c2 rat cardiomyoblasts. They found that glycofect performed better than linear polyethylenimine polymer because the former appeared to bypass lysosomes and was partially taken up in the Golgi apparatus (Fichter et al. 2013). These examples underscore the importance of avoiding lysosomal degradation to enhance the therapeutic efficacy of nanomaterials.

The intracellular trafficking of nanomaterials in polarized cells can lead to a special form of mobilization. In these cells, nanomaterials can end up on the opposite membrane in a process known as transcytosis (Tuma and Hubbard 2003). This route is important for the delivery of substances across capillary endothelial cells, especially across the blood brain barrier (BBB). The BBB is important because it separates the circulating blood from the brain extracellular fluid, posing a major hindrance to the successful delivery of therapeutics to the central nervous system (Agarwal et al. 2009; Pardridge 2007; Tiwari and Amiji 2006). In an attempt to overcome this barrier, Chang and coworkers decorated PLGA nanoparticles with transferrin, which resulted in an effective targeting to CvME and a 20-fold increase accumulation across the BBB (Chang et al. 2009). Harush-Frenkel and coworkers showed that both cationic and anionic polyethylene glycol-polylactide nanoparticles (89.8 ± 4 and 96.4 ± 3 nm, respectively) were internalized via CME into MDCK cells. Interestingly, the cationic nanoparticles efficiently crossed the cells via transcytosis and accumulated at the basolateral membrane, whereas the anionic nanoparticles accumulated in the degradative lysosomal compartments (Harush-Frenkel et al. 2008). This interesting finding was not explored further by these authors, yet merits additional examination. In a biologically inspired example, Georgieva and coworkers decorated nanoparticles with prion peptides to target CvME, and they successfully improved the ability of these nanoparticles to cross the BBB; prion proteins are known to bind to specific receptors and mediate transcytosis from the apical surface of brain endothelial cells (Georgieva et al. 2011). Collectively, these studies point out that in vitro studies can

be helpful in understanding and developing nanomaterials capable of solving intricate problems, such as crossing the BBB.

In some cases, the objective goes beyond targeting traditional intracellular sites. In these situations, different strategies must be used to reach these compartments, such as the nucleus. In one such strategy, Chen and coworkers pointed out that the nucleolin protein present at the surface could work as a DNA receptor and shuttle DNA nanoparticles from the membrane into the nucleus (Chen et al. 2008). Later, the same group demonstrated the feasibility of this mechanism in vivo by showing the expression of nucleolin on the apical surface of mouse airway epithelia, suggesting this could be a good target for nonviral gene delivery (Chen et al. 2011). Using a similar approach, Dam and coworkers used a DNA aptamer (AS1411) with high binding affinity to nucleolin to take advantage of the shuttling properties of nucleolin to efficiently target gold nanostars into the nucleus of cancer cells. They found that the nanoconstructs were close to the nuclear membrane and induced changes in nuclear shape. The authors suggested that these changes interfered with nuclear functions, which in turn increased caspase 3 and 7 activity (apoptosis), and also decreased cell viability (Dam et al. 2012). According to Wang and coworkers, titania nanotubes can cross the nuclear membrane, thereby reaching the nucleus of mouse neural stem cells. Given this finding, the authors suggested that these nanotubes can be used for delivery of DNA-targeting drugs (Wang et al. 2010). Although these strategies show promise for the delivery of cargo to the nucleus, care should be taken to evaluate potential toxic side effects associated with the delivery of these nanomaterials to the nucleus.

The cytotoxic effect of nanomaterials is the focus of the next discussion. It is well documented that surface modifications of nanomaterials can affect both their internalization pathway and their intracellular fate. An illustrative example of this behavior was demonstrated using cerium oxide nanoparticle derivatives. Their neutral derivatives were localized mostly in the cytoplasm of cells and hence did not elicit cytotoxicity to cancer cells. Conversely, the negative and positive derivatives that were localized in lysosomes did exhibit cytotoxic effects. The authors explained that the low pH of the lysosomes activated the oxidation of the nanoceria nanoparticles, thereby sensitizing tumors toward radiation therapy (Asati et al. 2010). In another example, several quantum dots were synthesized to extend their intracellular retention time, and the authors found that oxalate-transferrin quantum dots were able to delay their cellular removal both in vitro and in vivo. The authors suggested that these modified quantum dots could have a diverse range of applications, including diagnostic imaging, improved payload release, and decreased nanotoxicity (Wu et al. 2013). Although surface modifications of nanoparticles can be an effective strategy for improving nanomaterial uptake, this approach should be carefully rationalized. After nanoconjugation, the new nanomaterial can be internalized and processed by the cells in a different way than the naked nanomaterial or the free ligand. Indeed, Iversen and coworkers demonstrated that ricin alone was internalized by both dynamin-dependent and dynamin-independent endocytic pathways; however, the nanoconjugated form (ricin quantum dots) did not use these pathways (Iversen et al. 2012).

Since surface modifications of nanomaterials are a common strategy for controlling the endocytic pathway and consequently the intracellular fate of nanomaterials, this topic will be discussed in more detail in this section. Furthermore, recent new methodologies and findings have afforded more details on the intracellular trafficking of nanoparticles. Wang and coworkers have developed an interesting methodology to study intracellular trafficking of nanoparticles. Using dual-color nanoparticle pairs to measure size distribution within caveolae and assembly dynamics in living endothelial cells, they showed that in one caveolae, it is possible to find up to three 20-nm nanoparticles or two 40-nm nanoparticles (Wang et al. 2009). Details about how cells control intracellular trafficking were found using H89, an inhibitor of protein kinase A (PKA). Rehman and coworkers demonstrated that the modulation of PKA activity strongly affected the intracellular trafficking or CME internalization of both poly- and lipoplexes. The authors found that this inhibition channeled the lipoplexes to non-degradative compartments, resulting in a 2–3-fold increase in the transfection efficiency of branched polyethylenimine polymers in Hela cells (Rehman et al. 2011). These findings illustrate new approaches that can be used to help us better understand the intracellular trafficking of nanoparticles to achieve better outcomes. These advances represent the types of approaches that will be necessary to further improve the field of nanotechnology and to achieve its expected impact on medicine.

9.4 Cellular Metabolism of Nanodevices: Biodegradation and Elimination

Ideally, after nanomaterials enter cells and play their biological role, such as delivering drugs or genes, cells should be able to metabolize and eliminate these nanomaterials. Additionally, the metabolites generated should not be toxic. Indeed, nanomaterial metabolism and elimination should be carefully considered, since an optimal balance between lack of toxicity and therapeutic effect can be difficult to achieve. Therefore, studying the intracellular metabolism of nanomaterials is crucial for understanding their overall effect inside cells.

Typically, minutes after internalization, the maturation of the EE starts and the nanomaterials are sorted to different intracellular destinations, eventually ending up in lysosomes. This process begins with the acidification of the EE to a pH of 6.8–6.1, continues to a late endosomes (LE) with a pH of 6.0–4.8, and eventually reaches the lysosomal compartment equipped with degradative enzymes (e.g., proteases, esterases, phosphatases, nucleases, and lipases) and a pH of around 4.5 (Huotari and Helenius 2011). This lysosomal compartment is the limiting step for effective biological-based therapy, such as the delivery of peptides, proteins, or nucleic acids (Varkouhi et al. 2011; Won et al. 2009). For example, del Pozo-Rodríguez and coworkers found that solid lipid nanoparticles (281 $\pm$ 69 nm) used as a gene delivery system were inefficient because CME targets its DNA cargo to

lysosomal degradation in ARPE-19 cells (del Pozo-Rodríguez et al. 2008). Although CME is well known for targeting its contents directly to lysosomes, cargo from CvME and macropinocytosis eventually also reach lysosomal compartments (Dharmawardhane et al. 2000; Ekkapongpisit et al. 2012; Sahay et al. 2010a, b). In most cases, after internalization, nanomaterials must escape from endosomes to prevent cargo degradation within lysosomes. Therefore, several strategies have been used to improve endosomal escape (Varkouhi et al. 2011). Caracciolo and coworkers solved this problem by using a protamine/DNA complex coated with a lipid envelope made of cationic 1,2-dioleoyl-3-trimethylammonium propane (DOTAP). This ternary complex was advantageous in terms of endosomal escape and DNA release, resulting in a very efficient gene delivery system for different cell lines (Caracciolo et al. 2011). Another strategy is to take advantage of the drop in pH during endosomal maturation to trigger the release of cargo into the cytoplasm. Wang and coworkers developed gold nanoparticles that can release their content in an acidic environment; these particles efficiently delivered doxorubicin and inhibited the growth of multidrug-resistant MCF-7/ADR cancer cells (Wang et al. 2011a). Similarly, hydroxide nanoparticles successfully delivered methotrexate to cells and thus enhanced drug efficacy due to their anion exchange capacity in acidic pH (Oh et al. 2006). Although some strategies can be used to escape endosomes, lysosomal degradation still remains the limiting step in the efficient delivery of biological cargo.

Another cellular trafficking strategy is known as exocytosis. Exocytosis is used to expel nanomaterials from cells when nanomaterials resist destruction, even after exposure to numerous degradative enzymes, or when cells are exposed to large amounts of nanomaterials and thus need to avoid lysosomal overloading. Exocytosis is a biological process that is highly conserved across species and cell types. It is a process by which intracellular vesicles fuse with the plasma membrane and vesicle contents are released into the extracellular space. It is the major intracellular route for the delivery of proteins and lipids to the plasma membrane, making this process vital for many physiological processes, including membrane expansion during cell division, cell growth and cell migration, establishment of cell polarity, cellular communication, neurotransmission, and the secretion of hormones and cytokines (Ory and Gasman 2011).

Recently, exocytosis was linked to the chronic cytotoxic effects of nanomaterials by regulating their intracellular retention times. Typically, nanomaterials undergo internalization through endocytosis, then the endosomes containing the nanomaterials are transported by dyneins, microtubule-dependent motor proteins. Afterwards, a fraction of internalized nanomaterials can be found in lysosomes and/or transported to the perinuclear region by kinesins, another type of motor proteins. Finally nanomaterials can be sent out of the plasma membrane into the extracellular space via exocytosis. Bae and coworkers have described the same pathway for lanthanide-doped upconverting nanoparticles in HeLa cells, whereby nanoparticles avoid lysosomal degradation and result in high photostability and low cytotoxicity (Bae et al. 2012). Also in HeLa cells, Jiang and coworkers have found a similar pathway for zwitterionic quantum dot nanoparticles. However, these

authors found a significant fraction of their nanoparticles in lysosomes, while the remaining fraction was actively transported to the cell periphery and exocytosed with a half-life of 21 min (Jiang et al. 2010). This could indicate that cells try to avoid lysosomal overload, which eventually could result in leaking of lysosomal contents into the cytoplasm, resulting in cytotoxic effects. Even plant cells use exocytosis as a strategy to eliminate single-walled carbon nanotubes to minimize cytotoxicity (Serag et al. 2011). Johnston and coworkers used cellular imaging to show that polystyrene nanoparticles (20 nm) accumulated within distinct areas between adjacent hepatocyte cells, which the authors hypothesized represented bile canaliculi (Johnston et al. 2010). Together these studies point out that exocytosis is a universal process used by eukaryotic cells to minimize cytotoxicity.

As discussed before, surface modifications of nanomaterials can dramatically influence their biological fate inside cells and their targeting to the extracellular space. Gold nanoparticles decorated with different peptides followed different endocytic pathways and thus had a different exocytic profile in human endothelial cells (HUVECs). The authors showed that nanoparticles coupled to the peptide KPRQPSLP were reinternalized by cells after 4 h, whereas particles coupled to the peptide KATWLPPR were progressively exocytosed for a period of 6 h. Interestingly, these gold nanoparticles were internalized, processed by the cells and exocytosed, while still keeping their physicochemical properties (size and zeta potential), showing that these are nonbiodegradable nanoparticles (Bartczak et al. 2012). This finding reinforces the idea that nanoparticle surface modifications alter the way nanoparticles interact with the biological milieu, which subsequently determines their fate.

Another nanomaterial property that influences exocytosis is nanoparticle size. Smaller gold nanoparticles are more quickly exocytosed than larger ones. Dombu and coworkers found that cholesterol depletion in human bronchial epithelial cells increased endocytosis of polysaccharide cationic nanoparticles. Two hypotheses were proposed by the authors to explain the mechanism mediating these effects: (1) cholesterol depletion could lead to caveolae disruption, which in turn would trigger a compensatory effect via CME, or (2) cholesterol depletion could compromise nanoparticle exocytosis. To address this hypothesis, the authors followed nanoparticle exocytosis for up to 240 min in the presence and absence of filipin, which specifically binds to cholesterol. Surprisingly, filipin treatment diminished nanoparticle exocytosis, indicating that exocytosis of nanoparticles in these cells occurred via a cholesterol-dependent pathway (Dombu et al. 2010). Although the findings clearly demonstrate that exocytosis is an important mechanism in nanomaterial processing, further research is needed to determine the underlying mechanism of this process.

Until recently, it was believed that exocytosis of nanoparticles was independent of the permeability glycoprotein (P-gp) pathway (Jiang et al. 2010; Jin et al. 2009; Panyam and Labhasetwar 2003; Wang et al. 2011a). Although many nanoparticles have been developed to try to overcome P-gp-mediated drug efflux, a recent finding showed that nanoparticles can also be substrates for this transporter. Al-Hajaj and coworkers found that functionalized quantum dots (8–10 nm in size) were

eliminated from human liver Hep G2 cells and kidney Hek 293 cells via the P-gp transporter (Al-Hajaj et al. 2011). Here it is important to highlight that, the particles' size seems to play an important role in this process; therefore, it is highly unlikely that larger nanomaterials experience the same elimination pathway.

9.5 Mechanisms of Nanomaterial Cytotoxicity

Nanomaterials take advantage of special properties afforded at the nanoscale, such as large surface area, chemical reactivity, physical absorption ability, and quantum size effects, to perform unique functions. Nevertheless, once inside cells, these properties can bring undesirable effects, such as cytotoxicity. These effects can cause local damage that can be repaired by the cells, and they can also get out of control and compromise tissue or organ function, ultimately compromising human life. Thus, the fast development of nanotechnology over the last decades has raised concerns about the effects of these new nanomaterials on human health. Therefore, the evaluation of these effects has culminated into the creation of a new subdiscipline in nanotechnology, namely, nanotoxicology (Donaldson et al. 2004). The establishment of this new field has been marked by the publication "Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles" (Oberdörster et al. 2005) and the introduction of the *Nanotoxicology Journal* in 2007. After this, much attention has been paid to this topic, and several other reviews have since been published (Ai et al. 2011; Alkilany and Murphy 2010; Buzea et al. 2007; El-Ansary and Al-Daihan 2009; Hoet et al. 2004; Jian et al. 2012; Nel et al. 2006). Although studies in this field already existed before it was formally established, it is still not completely equipped with all the necessary scientific tools to properly evaluate all the possible toxic effects nanomaterials can elicit. Therefore, old techniques are being adapted, and new ones have been introduced to help evaluate the hazards and risks associated with nanomaterials (Hillegass et al. 2010; Hussain et al. 2009; Lai 2012; Lai et al. 2012; Love et al. 2012b; Marquis et al. 2009).

Nanomaterial toxicity can emerge from undesirable interactions in the cellular milieu, which can be driven by the physicochemical properties of the nanomaterials, such as retention time inside the cell, surface properties, and toxic metabolites. These undesired interactions can influence a number of cellular events that can lead to cytotoxic effects, such as morphological and structural changes (plasma membrane damage, alterations in cell morphology, and cytoskeleton defects), genotoxicity (gene-expression alterations, DNA damage, micronuclei formation, chromosomal aberrations), and biochemical alterations (oxidative stress, lipid peroxidation, Ca^{2+} release, caspase activation), which in turn can trigger different cellular responses (cell-cycle and proliferation irregularities, inflammatory cytokine production, diminution in mitochondrial function, activation of cell signaling pathways, autophagy, apoptosis, and cell death) (Lai 2012;

Love et al. 2012b; Soenen et al. 2011). Some examples of the most common nanomaterial toxic effects are discussed below.

9.5.1 Morphological and Structural Changes

The plasma membrane represents the first site of interaction for nanomaterials and cells and the first barrier that nanomaterials must overcome to reach their intracellular target. Although the main mechanism by which nanomaterials enter cells is by endocytosis, as already discussed, in some cases nanomaterials can apply a physical stress on the plasma membrane and disrupt it. A disruption in the integrity of the plasma membrane can directly compromise its role as a barrier, leading to intracellular leakage and cell death. For silver nanoparticles, the damage to the cell membrane was reported to be time and concentration dependent (Hussain et al. 2005; Mukherjee et al. 2012). Silver nanoparticles together with other metallic nanoparticles (gold and platinum) were also harmful to bacteria and fungi, basically by disturbing the bacterial wall or fungal membrane (Chwalibog et al. 2010). Silica nanoparticles damaged the plasma membrane and also membranes inside the cells, i.e., the mitochondrial membrane (Sun et al. 2011). This toxic effect appears to be related to particle size; smaller particles seem to have greater potential to cause damage to the plasma membrane. Kasper and coworkers found that small amorphous silica nanoparticles (30 nm) caused greater damage to the plasma membrane of lung epithelial cells (H441) when compared to larger ones (70, 300 nm) (Kasper et al. 2013). Similarly, Liu and coworkers showed that silver nanoparticles damaged plasma membranes at lower concentrations ($\geq$25 μg mL^{-1}) when compared with micro-sized silver particles (100 μg mL^{-1}) (Liu et al. 2011).

Nanomaterials can also disturb the cellular cytoskeleton. This deregulation can compromise chromosome segregation and cytokinesis, inhibiting cell division. Morphological changes in cells exposed to nanoparticles may be due to interferences with the structure and function of the actin cytoskeleton (Asharani et al. 2009). This concept was confirmed through specific staining of the actin cytoskeleton with rhodamine-labeled phalloidin. Gold nanoparticles induced aggregation and formation of dot-like structures of actin filaments in A549 cells (Wang et al. 2011b). Soenen and coworkers found that the actin cytoskeleton was affected by gold nanoparticles at lower levels (50 nM) than that needed to disturb tubulin networks (100 nM), suggesting that actin fibers are more sensitive to nanoparticle-induced deformations (Soenen et al. 2012).

9.5.2 Genotoxicity

Genotoxicity has raised concerns about the safety of nanomaterials. Manufactured nano-/microparticles, such as fullerenes, carbon black, and ceramic fibers,

irrespective of their size, induced genotoxic effects in A549 cells (Totsuka et al. 2009). Several reports have also described the genotoxic effect of silver nanoparticles. They appear to cause a wide variety of DNA damage, including DNA double-strand breaks, chromosomal aberrations, chromosomal fusions, and chromosomal fragmentation (AshaRani et al. 2009; Asharani et al. 2009; Kim et al. 2010; Liu et al. 2011). However, there are also particles that display low or no genotoxicity, as demonstrated by Pierscionek and coworkers when they used cerium oxide nanoparticles (5 and 10 μg mL^{-1}) and found that they did not cause any DNA damage or chromosomal changes in cultured eye lens epithelial cells (Pierscionek et al. 2009). Evidence from recent published reports suggests that nanomaterials are involved in mammalian mutagenesis and, possibly, carcinogenesis. Recently, Ng and coauthors published a review covering the latest findings on nanomaterial genotoxicity and the methodologies used in these studies (Ng et al. 2010).

9.5.3 Biochemical Alterations

The generation of reactive oxygen species (ROS) seems to be a central mechanism by which most nanomaterial toxicity is mediated (Markovic et al. 2007; Walker and Bucher 2009). Nanomaterials can generate ROS in different situations and in different cellular environments, either inside cellular compartments or outside in the cytoplasm. After being internalized, the accumulation of nanomaterials in lysosomes can contribute to ROS production. Chen and coworkers showed that iron oxide nanoparticles induced cytotoxicity in U251 cells when entrapped in lysosomes. The iron oxide nanoparticles catalyzed the production of hydroxyl radicals from H_2O_2 via peroxidase-like activity. Surprisingly, the same nanoparticles, when present in the cytosol, decomposed H_2O_2 through catalase-like activity (Chen et al. 2012). Some authors take advantage of the ROS production mediated by nanomaterials to fight cancer cells. Biologically synthesized silver nanoparticles have shown anticancer properties in Hela cells, and increases in intracellular ROS levels seem to play a key role in mediating this effect (Jeyaraj et al. 2013). Similarly, starch-coated silver nanoparticles were toxic to Hela cells by significantly increasing hydrogen peroxide and superoxide production (AshaRani et al. 2009). Sanpui and coworkers reduced silver nanoparticle side effects by impregnating them in chitosan-based nanocarriers. Using a considerably lower concentration of silver nanoparticles (330 ng mL^{-1} at IC_{50}), they efficiently induced apoptosis through ROS generation in human colon cancer cells (HT 29) (Sanpui et al. 2011). Although most publications report that the toxic effects of nanoparticles are mediated by generation of ROS, some particles also show ROS-scavenging properties. For example, cerium oxide nanoparticles can protect biological tissues against radiation-induced damage by protecting them from H_2O_2-induced cell damage (Karakoti et al. 2009). In addition to their free-radical scavenger activity, cerium oxide nanoparticles also increased the expression of superoxide

dismutase 2 (Colon et al. 2010). These findings suggest that even nanomaterials with seemingly toxic effects can have useful applications. Therefore, in addition to evaluating the cytotoxic effects of nanomaterials, the controlled production and choice of nanomaterial for performing a specific task are fundamental, since nanomaterial properties and interactions with the cellular environment can dramatically change their behavior.

9.5.4 Cellular Response

The nanomaterial toxic effects discussed here rarely occur alone. In fact a nanomaterial can trigger a multitude of events inside the cell. For example, the generation of ROS triggered by nanomaterials is commonly related to a rise in oxidative stress, leading to cell death, i.e., apoptosis. This toxic profile has been described for silver nanoparticles (Mukherjee et al. 2012) and silica nanoparticles (Sun et al. 2011). In addition, amorphous silica nanoparticles and titanium dioxide nanoparticles can induce cellular inflammatory responses (Kasper et al. 2013; Park et al. 2008). For example, Lunov and coworkers revealed the complex underlying mechanism of toxicity for amino-functionalized polystyrene nanoparticles in human macrophages. Specifically, nanoparticle internalization induced lysosomal rupture and leakage of active cathepsin B to the cytosol, which in turn elicited mitochondrial damage and production of ROS. Subsequently, the accumulation of mitochondrial ROS led to oxidation of the redox-active thioredoxin (TXN) protein that plays an important role in oxidative stress. Meanwhile, thioredoxin-binding protein (TXNIP) was released, which resulted in inflammasome activation and IL-1β production (Lunov et al. 2011). This study illustrates the complexity of cellular responses to nanomaterials and the importance of examining nanomaterial toxicity using different mechanisms.

Autophagy has also emerged as a mechanism to regulate nanomaterial toxicity (Stern et al. 2012). The macroautophagy pathway (herein autophagy) is a highly conserved biological process that involves the sequestration of proteins, lipids, and organelles, followed by their degradation within double-membrane structures called autophagosomes (Kroemer et al. 2010). Li and coworkers showed that gold nanoparticles create an oxidative environment in MRC-5 human lung fibroblasts. The authors suggested that this oxidative environment could affect the regulation of cellular stress response mechanisms and, at the same time, induce the formation of autophagosomes, as a possible attempt to protect the cell from oxidative stress (Li et al. 2010). Ma and coworkers demonstrated that gold nanoparticles compromised lysosomal activity by alkalinization of lysosomal pH in normal rat kidney (NRK) cells. As a result of this, there was an accumulation of autophagosomes caused by the blockade of the autophagic flux (Ma et al. 2011). These studies suggest that autophagy is not only involved in nanomaterial toxicity but also influenced by it. Ultimately, autophagy can be considered a cellular response to foreign bodies (Zabirnyk et al. 2007).

It is worth noting that even without inducing cytotoxic effects, nanomaterials can impact normal biological processes in eukaryotic cells, such as cell signaling and cellular communication. Comfort and coworkers showed that silver nanoparticles reduced Akt and Erk signaling, while gold nanoparticles significantly diminished p-Akt and p-Erk levels and inhibited Akt activity. The authors showed that these alterations to cellular functions occurred both at the protein and genome level (Comfort et al. 2011). Another biological process that can be affected by nanomaterials is cellular communication. Love and coworkers observed that although gold and silver nanoparticles did not alter the viability of murine adrenal medullary chromaffin cells, they did alter their cellular communication. In addition, these authors showed that gold nanoparticles also interfered with cellular adhesion (Love et al. 2012a). Nonporous SiO_2 nanoparticles can also disrupt exocytosis in primary culture mast cells plus cause significant affects on cell viability by inducing hemolysis (Maurer-Jones et al. 2010). These findings support a body of work showing that distinct nanoparticles, such as nonporous SiO_2, gold, or silver nanoparticles, do disrupt the process of exocytosis, typically altering the number of molecules and release kinetics from vesicles (Love et al. 2012a; Maurer-Jones et al. 2010). These alterations in cellular metabolism might be considered subtle and remain unnoticed, since they do not lead to cell death. Nevertheless, they can be very important for some tissues, such as exocytosis in neuronal function and with long time exposure to nanomaterials, as in cell signaling disorders related to chronic diseases. These types of studies can also provide valuable information about nanomaterial toxicity.

9.6 Conclusions and Perspectives

It is tempting to think about a future where nanomaterials can solve diverse types of human problems. Although the first generation of nanoparticles is currently being approved for human use, there is still a long way to go. Over the last several decades, we have made significant progress in the area of cellular internalization and intracellular trafficking of nanomaterials, but broad generalizations still cannot be made. New findings can potentially address this limitation, but still we have to answer some questions: Why do different cells internalize the same nanomaterial in different ways or what makes it a cell type-dependent process? How do cells control intracellular trafficking of nanomaterials? What are the underlying mechanisms that nanomaterials use to escape endosomes? Are there other endocytic pathways? Do endocytic pathways converge on the same compartment?

Current knowledge suggests that broad generalizations cannot be made in this field. Perhaps the variety of cell types and their specific functions do not align with this idea. It may be better to find a balance between understanding the intrinsic characteristics of nanoparticles and knowing the changes that we can make to mediate the changes that we are looking for. The journey toward finding a "magic bullet" has left some things overlooked. For example, the field has focused

on metallic nanomaterials, although a better approach might be to consider biodegradable materials, such as polymers and lipids. These materials could be used in new applications because of their intrinsic characteristics and thus give us a wider range of tools for nanomedicine. In addition, it seems that exocytosis plays a more important role in intracellular trafficking of nanomaterials than we initially thought. The field has just started looking in this direction, but we can already formulate new interesting questions from this initial knowledge. For example, does the exocytosed nanomaterial undergo internalization again? Does it take the same route?

As the development of new nanomaterials increases and their human applications become reality, the concerns about the potential hazards of nanomaterials also increase. This led to the establishment of a new field, nanotoxicology. Although this field has just emerged, it has many important issues to address. On one side it faces a great deal of pressure due to the fast growing area of nanotechnology, on the other side, it has the responsibility to examine and guarantee safety for human health and the environment. A potential strategy to address these pressures might be to improve in vitro toxicity assays and studies to evaluate the risk imposed by nanomaterials. Undoubtedly, there are limitations to the in vitro assessment of nanomaterial toxicity. Although, with additional studies these limitations can be addressed and guided by in vivo studies that can lead to better and more specific protocols. In turn, the knowledge gained from in vitro studies can help to predict or anticipate new targets to better define strategies for in vivo evaluations. In addition, distinguishing between normal, transient and real adverse effects of nanomaterials will help. Ultimately, a broader examination is paramount to understanding the impact of nanoparticles on cellular metabolism. These evaluations can help us to determine if nanomaterials will have an affect on chronic or debilitating diseases, such as multiple sclerosis and cancer.

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