Nanoparticles: Cellular Uptake and Cytotoxicity

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Abstract

Understanding the interactions of nanoparticles (NPs) with cells and how these interactions influence their cellular uptake is essential to exploring the biomedical applications of NPs, particularly for drug delivery. Various factors, whether differences in physical properties of NPs or variations in cell-membrane characteristics, influence NP-cell interactions and uptake processes. NP-cell membrane interactions may also influence intracellular trafficking of NPs, their sorting into different intracellular compartments, cellular retention, and hence the efficacy of encapsulated therapeutics. A crucial consideration is whether such interactions might cause any toxicity,

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starting with how NPs interact in transit with the biological environment prior to their interactions with targeted cells and tissues. Understanding the effects of various NP characteristics on cellular and biological processes could help in designing NPs that are efficient but also nontoxic.

Keywords
Polymers • Drug Delivery • Transport • Nanocarriers • Biocompatibility

Abbreviations

AFM	Atomic force microscopy
AR	Aspect ratio
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptides
CTAB	Cetyltrimethylammonium bromide
DMAB	Didodecyldimethylammonium bromide
DTAB	Dodecyltrimethylammonium bromide
HIV	Human immunodeficiency virus
MTs	Microtubules
NPs	Nanoparticles
PLGA	Poly (D, L-lactide co-glycolide)
RISC	RNA-induced silencing complex
RNAi	RNA-interference
siRNAs	Small interfering RNAs
SNPs	Silica NPs
TAT	*trans*-activating transcriptional activator

5.1 Introduction

Nanoparticles (NPs) with unique physical characteristics such as size, shape, and surface chemistry or that have been modified with different targeting ligands are designed and optimized to explore their use in various biomedical applications, particularly for drug delivery [1, 2] or imaging [3, 4]. The critical issue is to understand NP-cell interactions: for certain applications (e.g., gene therapy) [5], it may be desirable that NPs interact more efficiently with cells and more readily become internalized, whereas for other applications (e.g., vascular imaging agents), it may be necessary to minimize NP-cell interactions [6]. NP-cell interaction is a very dynamic process and depends on physical characteristics of NPs, as well as on cell-membrane properties [7, 8]. Physical characteristics of NPs such as shape, size, surface charge, or the presence of cell-penetrating peptides/targeting ligands on the NP's surface may influence NP-cell interactions. Similarly, cell-membrane properties such as membrane fluidity, type of receptors, receptor density, and recycling rate of receptors may influence NP-cell interactions and internalization [9]. Furthermore, NP-cell membrane interactions could determine the pathway by which uptake of NPs occurs, as well as their intracellular sorting into different cellular compartments and retention in the target area, which eventually could influence the efficacy of encapsulated therapeutics. In other cases, NPs are modified with hydrophilic polymers to minimize NP-cell interactions [10]. NP-cell membrane interactions are also being studied to understand NP-mediated toxicity. Therefore, it is timely to review parameters that influence NP-cell interactions, which could help in designing NPs that are efficient but nontoxic.

5.2 Mechanisms of Cellular Uptake

The mechanisms via which NPs enter cells are determined to a great extent by physical and interfacial characteristics of NPs, their interactions with the biological environment, and cell-membrane properties. NP size, shape, and surface characteristics (particularly charge and hydrophobicity) can influence the cellular uptake pathways [11, 12]. Furthermore, the interactions of NPs with cells may depend on conjugated ligands and cell-surface receptors for receptor-mediated uptake [13]. In addition, cell type and the nature of the cell's plasma membrane such as membrane fluidity, receptors, etc., can influence NP-cell membrane interactions and hence the uptake pathway. Several pathways for uptake may be used simultaneously, although with varying efficiency. Below we describe the common pathways by which NPs are internalized by cells.

5.2.1 Pinocytosis

Pinocytosis is the internalization of extracellular fluid and its content by cells and is subdivided into micro- or macropinocytosis, depending on the size of the cell-membrane invagination that traps the extracellular fluid. This pathway of cellular internalization of NPs can occur without direct interaction of NPs with the cell membrane because the bulk extracellular fluid is internalized [14]. However, NPs that interact with cell membranes have higher uptake through this mechanism than NPs that do not. Because of the small size of the cell membrane invaginations, pinocytosis is the predominant pathway for the uptake of large NPs and microparticles. Micropinocytosis occurs in almost all cells, whereas macropinocytosis occurs in specific cell types, e.g., immature dendritic cells [15, 16].

5.2.2 Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis (CME) is a cellular uptake mechanism that involves the formation of clathrin-coated endocytotic vesicles that are usually ~100 nm in diameter [17, 18]. Also known as receptor-mediated endocytosis, this uptake process is initiated by the binding of a ligand to its receptors on the cell membrane (Fig. 5.1). CME is the mechanism cells use to internalize ligand-conjugated NPs and it can be used to target NPs to specific cells [19, 20]. CME also becomes important when opsonins bind to NPs in a biological medium. An example is the opsonization of NPs by complement, that is, the group of proteins that are recognized by receptors on macrophages [21]. Another requirement to induce endocytosis is the size and amount of the cargo. It would take only a few large NPs to induce endocytosis, but cluster of many small NPs to induce CME [22].

5.2.3 Caveolae-Dependent Endocytosis

Caveolae are vesicles formed by cell-membrane invaginations that are 50–100 nm in diameter [23]. Caveolar vesicles enclose predominantly sphingolipids, cholesterol and caveolin (the predominant protein in caveolae) and bind to the associated protein to form microdomains, which dictate the cargo that is transported [24–26] (Fig. 5.1). These microdomains, which can con-

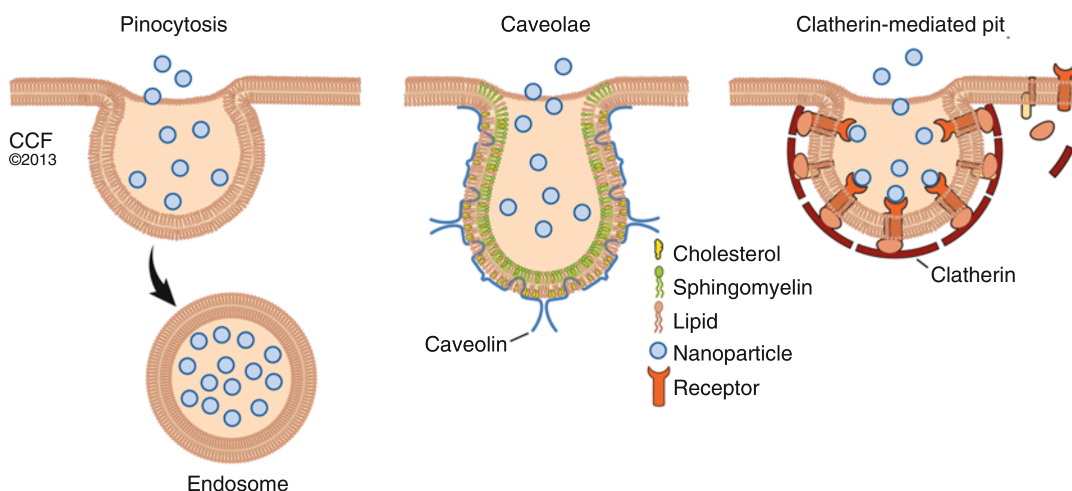

Fig. 5.1 Schematic of different cellular uptake mechanisms for NPs. The mechanisms of cellular uptake are determined by the physical characteristics of NPs. NPs with targeting ligands are generally internalized by clathrin-mediated endocytosis. Caveolae-mediated endocytosis is responsible for internalization of anionic NPs, whereas pinocytosis is the mechanism of choice for large NPs and microparticles

tain cationic lipids like sphingomyelin (whose polar domain contains an amine group), can interact with and mediate the endocytosis of anionic NPs like pegylated gold NPs where NP modified with polyethylene glycol [27, 28]. Caveolae-dependent endocytosis is the predominant pathway of uptake of NPs in endothelial and muscle cells [25, 29].

5.3 Interaction of NPs with Cells

5.3.1 Effect of Size and Shape of NPs on Cellular Uptake

The size of NPs influences their interaction with cell membranes and ultimately their intracellular uptake. NPs that are about 50 nm in diameter are generally taken up more rapidly by cells than larger NPs [30]. This preferential uptake of small NPs occurs because of the size of clathrin-coated pits and caveolar cell-membrane invaginations (50–200 nm in diameter) [31]. NPs smaller than 25 nm, on the other hand, may be too small, and hence large numbers are required to induce CME and caveolae-mediated endocytosis. For NPs smaller than 25 nm, pinocytosis is the preferred mechanism for uptake.

Although the size of NPs influences cellular uptake, this process is also influenced by cell type. Embryonic fibroblasts preferentially internalize single-walled nanotubes and gold NPs that are 25 nm rather than larger NPs. Epithelial cells, on the other hand, prefer 50 nm gold NPs over 25 nm or 70 nm gold NPs [27, 32]. These differences in cellular uptake are dependent on the predominant pathway for cellular uptake of each cell type. Cells in which cellular uptake is predominantly through macropinocytosis have a greater uptake of NPs >200 nm than cells processed via CME- or caveolae-dependent endocytosis.

Unlike inorganic NPs, which are relatively uniform in size, polymeric NPs, dendrimers and liposomes are polydisperse, which makes studying the impact of particle size on cellular uptake difficult [33]. This inherent polydispersity of polymeric NPs also affects our ability to predict the behavior of NPs interacting with cells.

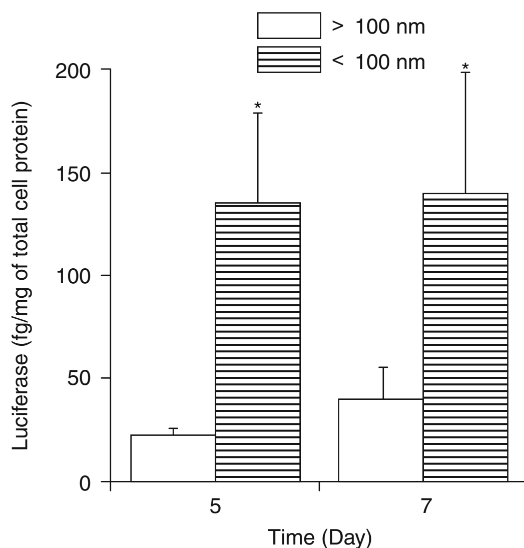

Fig. 5.2 Gene expression by small and large NPs fractionated from a single formulation in HEK-293 cells. The size of NPs influences the extent of gene expression. Cells transfected with NPs that are <100 nm in diameter have higher luciferase protein than cells transfected with NPs >100 nm (Reproduced with permission from Ref. [35]. Copyright 2002, Elsevier Ltd.)

However, even with these NPs, those with smaller mean particle size have a higher cellular uptake. An example is albumin NPs, where NPs with mean hydrodynamic diameters of 40 nm have greater cellular uptake than those with diameters of 100 nm [34]. Studies in our laboratory have also shown that NPs of <100 nm have greater transfection efficiency than larger (200 nm) NPs that were fractionated from the same formulation (Fig. 5.2). In this study, both NP populations showed similar uptake per weight, but the number of NPs internalized for the same weight would be greater for NPs of <100 nm than for NPs >200 nm, which could be the reason for higher gene expression with small NPs than with large ones [35]. The results thus suggest that NPs <100 nm are better for transfection than larger sized NPs.

When analyzing the cellular uptake of NPs on the basis of size, it is also important to determine the propensity of NPs to aggregate. Although most cellular uptake studies assume that cells interact with single NPs, some NP formulations (particularly those with a cationic surface charge)

aggregate in the presence of protein, making NP aggregation an important factor to consider when studying cellular uptake [36].

The shape of NPs influences how easily they are taken into cells, with rod-shaped gold NPs having a lower cellular uptake than spherical ones [37]. Among NPs of similar surface charge and diameters, the aspect ratio (AR), which defines the proportion between width and height of NPs, is more significant than size in predicting cellular uptake. NPs with an AR of 4 have been shown to have lower uptake by cells than those with ARs of 1 or 2 [27, 38]. These differences are explained by the kinetics of cellular uptake. It takes longer for the cell membrane to wrap around rod-shaped NPs than spherical NPs, and more rod-shaped NPs are required to induce endocytosis, in contrast to spherical NPs [7].

5.3.2 Effect of Surface Characteristics and Charge of NPs on Cellular Uptake

Surface characteristics of NPs can significantly influence their interactions with the cell membrane and hence their internalization pathways [39]. Surface characteristics and charge can influence (1) NP interactions with an anionic cell membrane [40] and (2) adsorption of proteins onto NPs, both of which affect NP-cell membrane interaction [3, 41–43]. A cell membrane is anionic because of the anionic head group of phospholipids and the presence of carbohydrates such as sialic acid [44, 45]. Even with their anionic surface charge, NPs interact with cell membranes and are taken up by different cells. This is because of interactions of anionic NPs with cationic lipid domains in the cell membrane [24, 46]. Because anionic NPs interact with lipid domains, their uptake usually involves caveolae-mediated endocytosis and not the classical CME pathway.

Much less studied aspects of cellular uptake of NPs are the roles of adsorbed proteins on anionic NPs and their role in cellular uptake. Opsonins such as complement and immunoglobulins adsorb onto anionic NPs and induce cellular uptake by CME instead of caveolae-mediated uptake [47]. The surface charge on NPs is conferred by the surface chemistry, and the nature of the chemical groups coating the NPs can influence NP-cell interaction [43]. Dimercaptosuccinic- and heparin-coated NPs both have anionic surface charges but show different interactions with cell membrane and subsequent uptake [48, 49].

Neutral NPs exhibit limited cellular uptake and are useful in applications where nonspecific interactions of NPs with cells and their subsequent cellular uptake is not desired. Modifying NPs with hydroxyl functional groups can attain a neutral surface charge [50]. The use of zwitterions can also impact neutral surface charge onto NPs [51]. These modifications decrease the interaction of NPs with plasma membrane and ultimately decrease cellular uptake.

Unlike anionic NPs, cationic NPs can directly bind to a cell's negatively charged plasma membrane [52]. This binding can be to the anionic head group of lipids or to other negatively charged groups on the cell membrane, such as monosaccharide sialic acid. Once they interact with the cell membrane, cationic NPs can induce internalization by CME. Because of their interaction with cell membranes and rapid endocytosis, cationic NPs serve as the basic platform for gene delivery and other applications that require rapid cellular internalization [50, 53, 54]. To obtain a cationic charge, positively charged polymers like chitosan and cationic emulsifiers like didodecyldimethylammonium bromide (DMAB) are being used in NP formulations [55, 56].

The molecular structure of surface modifiers also plays important roles in cellular interaction and uptake of NPs. A classic example is with cationic NPs; it is generally thought that it is the cationic surface charge of NPs that causes their interaction with the cell membrane. However, recent studies in our laboratory have shown that the molecular structure of the cationic surface modifier also influences interactions with the cell membrane and NP uptake. NPs modified with dichain cationic emulsifiers, DMAB, or single-chain cetyltrimethylammonium bromide (CTAB) and dodecyltrimethylammonium bromide (DTAB) showed different interactions with model

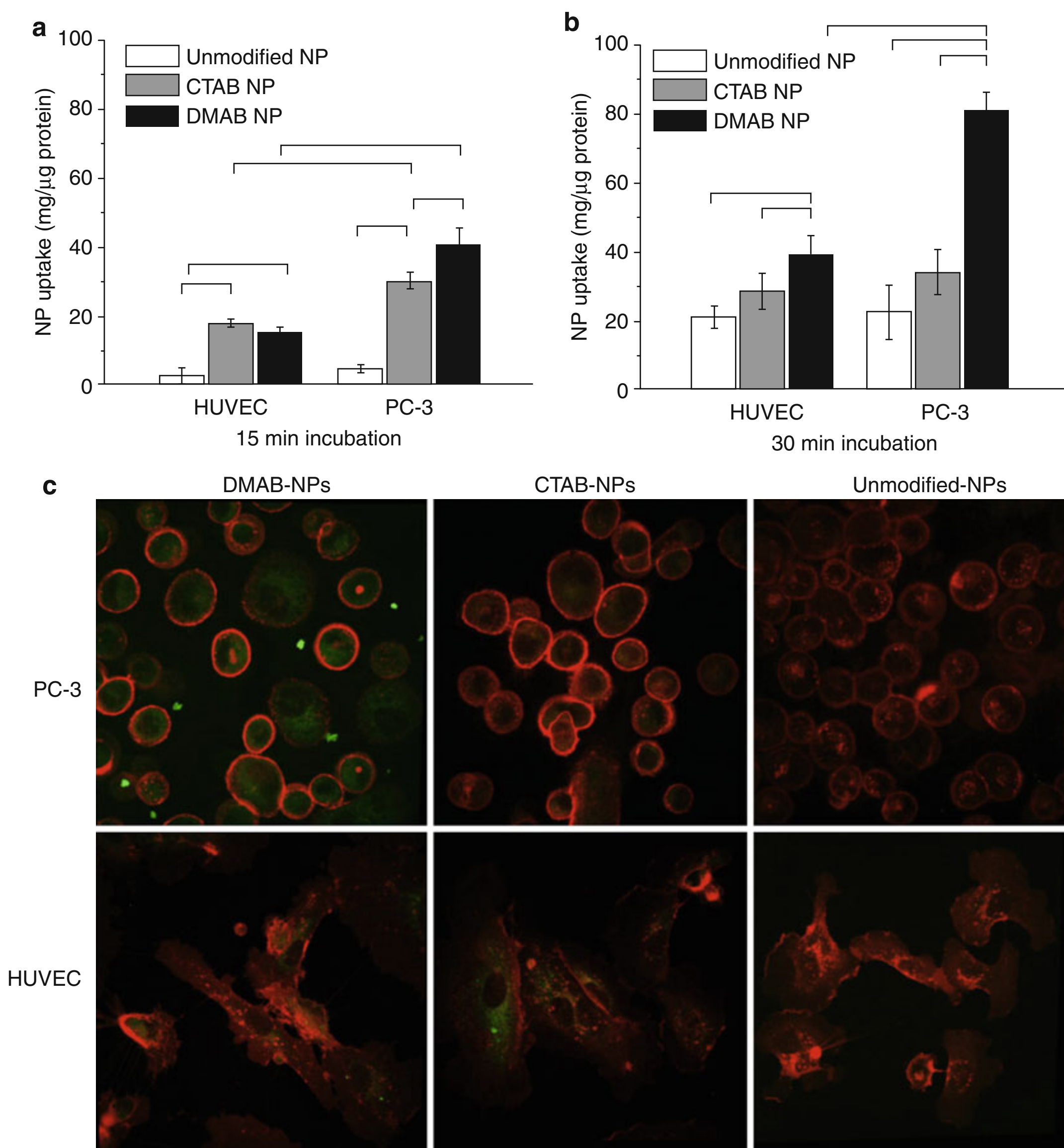

Fig. 5.3 The molecular structure of surfactants at the NP-cell interface influences cellular uptake of NPs, which is also cell-line dependent. Cellular uptake of unmodified, DMAB- and CTAB-modified NPs by human umbilical vascular endothelial cells and prostate cancer (PC-3) cells after 15 min (**a**) and 30 min (**b**) of incubation. (**c**) Confocal microscopy images of human umbilical vascular endothelial cells and PC-3 cells 30 min after incubation, with NPs with green and red representing NPs and cell membranes, respectively (Reproduced with permission from Ref. [59]. Copyright 2013, Elsevier Ltd.)

cell membranes. Dichain DMAB-modified NPs exhibited greater interaction and cellular uptake than single-chain, CTAB- or DTAB-modified NPs, although both formulations have a similar cationic zeta potential (Fig. 5.3). It has been proposed that DMAB, with its two hydrophobic chains, engages in greater interaction with the cell membrane than the single chains of CTAB and DTAB. Recently, we demonstrated that DMAB-modified NPs have greater biophysical interactions with prostate cancer cell-membrane lipids than normal human umbilical vascular

epithelial cell-membrane lipids. This selectivity of interaction leads to greater efficacy with p53 gene-loaded DMAB-modified NPs than unmodified NPs in tumor growth inhibition in a prostate cancer model [57–59].

5.3.3 Effect of Active Targeting on Cellular Uptake

To aid cellular internalization of NPs, particularly those with anionic and neutral surface charges, several targeting ligands have been used [60]. These ligands, e.g., transferrin, bind to receptors in the cell membrane and induce CME [61]. Because this method depends on the presence and number of target receptors, efficiency of targeted NPs is cell dependent and allows investigators to target specific cells in which receptors are overexpressed. In prostate cancer cells that overexpress transferrin receptors, transferrin-conjugated NPs demonstrate greater cellular uptake and are efficacious at inhibiting the growth of prostate tumors. In our studies, transferrin-conjugated NPs not only demonstrated greater cellular uptake but also showed sustained intracellular retention. Furthermore, direct intratumoral injection of paclitaxel-loaded transferrin-conjugated NPs demonstrated significantly greater tumor growth inhibition compared with unconjugated NPs, suggesting a greater degree of intratumoral retention of conjugated than unconjugated NPs, presumably due to interactions of conjugated NPs with transferrin receptors [62, 63] (Fig. 5.4).

Cell-penetrating peptides (CPPs), which facilitate cellular internalization of peptide-modified NPs, do not depend on receptors for cellular internalization. CPPs are rich in the positively charged amino acids arginine and histidine, which allows them to directly penetrate the cell membrane and become internalized [64]. However, because CPPs do not depend on receptors, they may not be cell specific [65]. An example of such CPPs is *trans*-activating transcriptional activator (TAT) peptide, derived from human immunodeficiency virus (HIV), which has shown efficacy in transporting NPs into different cells

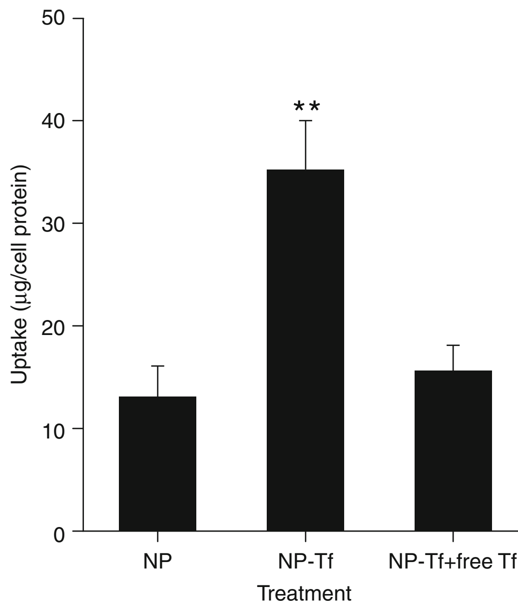

Fig. 5.4 Uptake of transferrin-conjugated and -unconjugated NPs by prostate cancer (PC-3) cells. Transferrin-conjugated NPs show greater cellular uptake than unconjugated NPs. Uptake of conjugated NPs is inhibited in the presence of an excess of free transferrin ligand, confirming uptake of transferrin-conjugated NPs is via receptor-mediated endocytosis brought about by transferrin receptors that are overexpressed on PC-3 cells (Reproduced with permission from Ref. [62]. Copyright 2004, John Wiley and Sons)

[66]. TAT-peptide-conjugated NPs have been particularly effective in transporting NPs into the brain, as they can cross the blood–brain barrier [67, 68].

In a similar manner as untargeted NPs, cellular uptake of conjugated NPs is affected by the size of NPs. Antibody-conjugated NPs of 25–50 nm in diameter have a higher cellular uptake than larger NPs [69]. For CPP-conjugated NPs, which directly penetrate the cell membrane, the effect of size becomes particularly significant as size increases [70]. This size limitation is due to the size of the clathrin-coated vesicles involved in their internalization.

Apart from NP size and the number of targeting ligands on NPs, the shape of NPs can influence their cellular uptake. In breast cancer cells that overexpress the *HER2/neu* receptor, nontargeted spherical NPs have been reported to show higher cellular uptake than rod- and

disk-shaped NPs, whereas when modified with antibodies to *HER2*/*neu*, nanodisks and nanorods show higher uptake than nanospheres [71]. This and other studies demonstrate that the shape of NPs can enhance cellular uptake of targeted NPs [72].

Despite the effectiveness of active targeting, NPs can be rendered ineffective when they are introduced into a biological medium. Depending on the physical characteristics of NPs, their surfaces (including conjugated ligands) can be covered with opsonins, making the ligand ineffective at initiating cellular internalization. This drawback can be overcome by using a linker to move the targeting ligand from the NP surface and away from the opsonins. The issue could also be overcome by designing NPs that are less opsonized [73].

5.4 Techniques for Studying NP-Cell Interaction and Cellular Uptake

5.4.1 Models for NP-Cell Interaction and Cellular Uptake

Understanding NP-cell interactions is important for designing NPs that either escape cellular uptake or are taken up efficiently by cells, depending on their applications. Studying this interaction is very difficult in whole-cell systems. Thus, different techniques using model cell membranes have been developed.

5.4.1.1 Lipid Monolayer

This method involves forming a lipid monolayer on an aqueous medium in a Langmuir-Blodgett instrument. Studies using this technique provide information about how strongly NPs interact with the head group of the lipids. Use of this method also helps determine if NPs will disrupt the cell membrane to enter the cell. When used in conjunction with atomic force microscopy (AFM), films from Langmuir-Blodgett interactions can identify interaction of NPs with lipid domains in the cell membrane [74, 75].

5.4.1.2 Supported Lipid Bilayers

A lipid bilayer can be formed with mica or silica wafers as a substrate or support to study interactions with NPs and drugs. The interaction between the lipid bilayer and drug/NP can be analyzed with AFM or other analytical techniques such as Fourier transform infrared resonance and X-ray photoelectron spectroscopy [76].

5.4.1.3 Liposomes

Liposomes are lipid vesicles formed to exclude the hydrophobic tails of lipids from aqueous phases and can be unilammelar or multilammelar. Multilammelar liposomes with two layers best mimic the cell membrane and are useful for studying the permeability of different NPs into the cell membrane. In conjunction with spectroscopic methods, the efficiency of NPs to cross lipid bilayers can be studied systematically [77].

5.4.2 Techniques for Studying Cellular Uptake

Several techniques are used to monitor the cellular uptake of NPs. Most of these are imaging or spectroscopic techniques that determine the cytoplasmic localization of NPs. To study the mechanism for uptake of NPs, various inhibitors of pathways of uptake have been used. These inhibitors help to elucidate different pathways cells use to internalize NPs and how the predominant pathway can change vis-à-vis the physical characteristics of NPs. A few of the commonly used inhibitors and the pathways/mechanisms they block are listed in Table 5.1. The NP uptake study is carried out at 4 °C or in the presence of metabolic inhibitors to determine if the uptake process is energy dependent, such as via endocytosis.

5.5 Intracellular Localization of NPs

Following cellular uptake of NPs, the next important question is where the NPs localize within cells. Where they finally localize affects the ther-

Table 5.1 Commonly used inhibitors to study mechanisms of NP uptake

Name of inhibitor	Pathway inhibited	Uptake mechanism(s) affected
Nocodazole	Polymerization of microtubule	Clathrin-mediated endocytosis [78]
Cytochalasin A	Polymerization of actin	Caveolae-mediated endocytosis [79]
Chlorpromazine	Reversible translocation of clathrin from cell membrane	Clathrin-mediated endocytosis [80]
Genistein	Tyrosine kinase inhibitor	Caveolae-mediated endocytosis [81]
Lovastatin	Cholesterol synthesis	Clathrin- and caveolae-mediated endocytosis and macropinocytosis [22]

apeutic/medical function of NPs, as well as cytotoxicity. After endocytosis, NPs are typically contained within endosomal vesicles, which have an internal pH of ~5 [82]; these vesicles mature into late endosomes before fusing with lysosomes, at which point they are subjected to digestive enzymes and degradation. This process limits the effective delivery of many therapeutic agents to intracellular targets, other than the lysosomes. Therefore, endosomal escape is often a critical step in intracellular trafficking of NPs and subsequent targeting to appropriate subcellular compartment(s). Intracellular targets include the cytoplasm (to deliver, for example, small interfering RNAs [siRNAs] or glucocorticoids), the nucleus (for delivery of DNA and DNA-intercalating agents, such as doxorubicin), mitochondria (anti-oxidants or mitochondrial DNA), or other compartments.

5.5.1 Strategies for Endolysosomal Escape of NPs

Mechanisms of endolysosomal escape must be strategically designed into NPs. A widely used approach takes advantage of the “proton-sponge effect,” which involves NPs having a high buffering capacity and the flexibility to swell when protonated. This is the typical mechanism via which cationic polymers, such as polyamines (polyethylenimine and polylysine are among the most common) escape the endosomal compartment [50, 54, 83]. These polymers have a strong buffering capacity, in the pH 5–7 range, and thereby prevent acidification of the endosomes by acting as “proton sponges.” Protonation induces the flow of ions (protons and Cl^-) and water (osmotic swelling) into the endosome, which subsequently causes rupture of the endosomal membrane and release of the polymeric complexes/NPs.

An extension of the proton-sponge effect is the “umbrella effect,” whereby the polymer unfolds from a collapsed to an extended state upon protonation at low pH (pH 5–6). The resulting increase in volume and space contributes to endosomal escape of the NP [83, 84]. NPs formulated from cationic lipids (lipoplexes) are able to escape from endosomes due to their interactions with the anionic phospholipids of endosome membranes. When lipoplexes are endocytosed, an electrostatic interaction occurs between the lipids of the lipoplex and the anionic lipids of endosomes facing the monolayer of the endosome, which consequently destabilizes the endosomal membrane by causing it to flip-flop. The cargo of the lipoplex is then released into the cytoplasm. This process can be controlled and enhanced based on the molecular structure of the lipids used in the lipoplex and the presence of lipids that facilitate the adoption of a nonbilayer structure (for example, dioleoylphosphatidylethanolamine and cholesterol) [83]. The use of cationic NPs is limited by their cytotoxicity, which relates to their mechanisms of entry into the cell as well as endosomal escape. Cationic NPs cause more pronounced disruption of the plasma membrane as well as mitochondrial and lysosomal damage compared with anionic NPs [50]. The strategy used by NPs formulated from cationic lipids is also employed by anionic NPs capable of charge reversal in the acidic endolysosome. In our study, we demonstrated that poly (D, L-lactide co-glycolide) (PLGA) NPs, which are anionic at physiologic pH (pH 7.4), undergo charge reversal in acidic pH of endosomes (pH ~5) and become

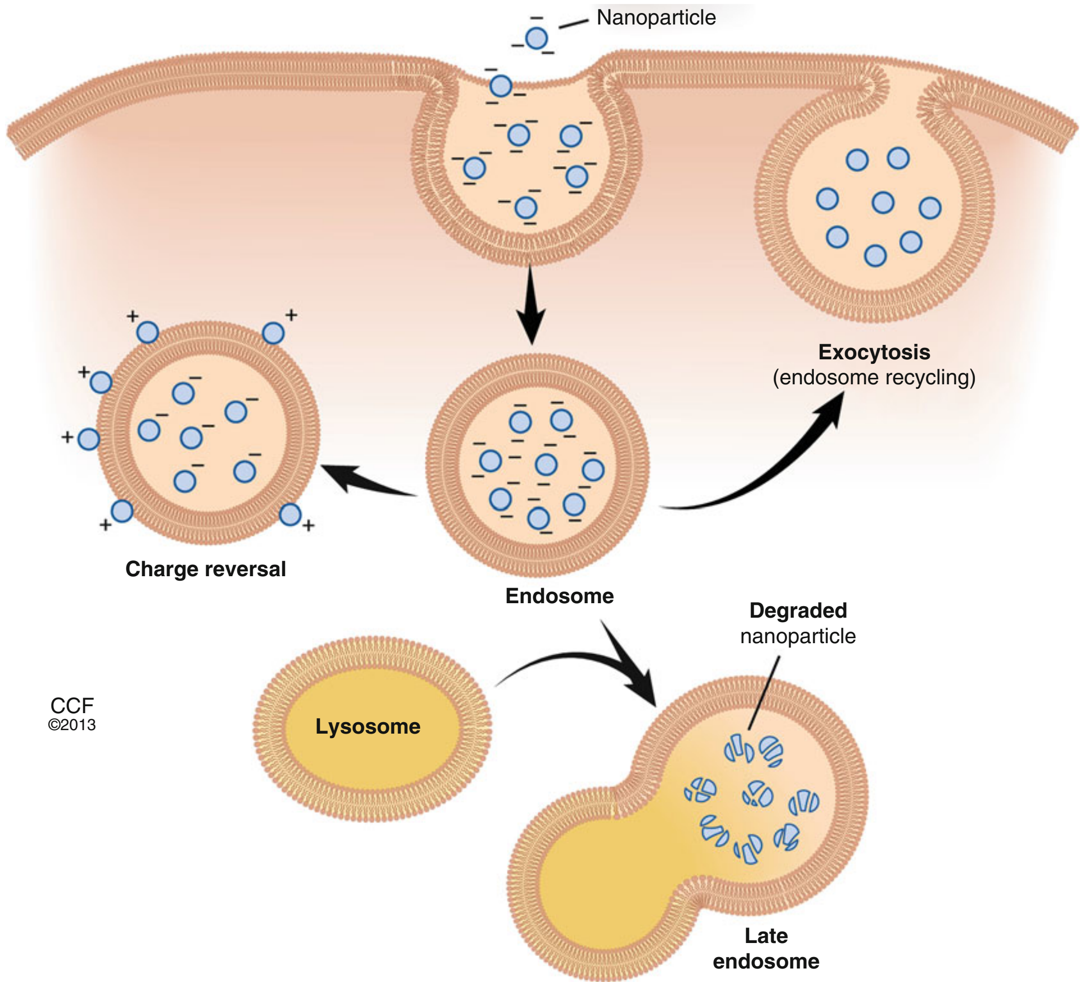

Fig. 5.5 Schematic of intracellular uptake and endosomal escape of NPs, depicting charge reversal in acidic pH (pH ~5) of endosomes. NPs that exhibit a pH-dependent charge reversal, such as poly (D, L-lactide *co*-glycolide) (PLGA) NPs, can escape the endosome by charge reversal. In the acidic condition of the secondary endosomes, anionic PLGA-NPs become cationic and interact with endosomal lipids, allowing their escape

cationic. This selective cationization of NPs in endosomes causes NPs to interact with the anionic endosomal membrane and escape into the cytoplasm. In that study, we demonstrated NP localization into the cytoplasmic compartment at as early as 10 min following incubation of cells with NPs, suggesting their rapid endosomal escape [85] (Fig. 5.5).

Agents that enhance endosomal escape, based on those employed by viruses and bacteria, have been used by researchers in the formulation of NPs. Fusogenic peptides have been incorporated onto NPs and used to destabilize the endosomal membrane. Membrane fusion plays an important role in cellular trafficking and endocytosis. Many viruses have membrane peptides, which undergo conformational change in response to a change in pH. These conformational changes allow the viral membrane to fuse with cellular membranes, including the lipid bilayer (for cell entry), as well as endosomal membranes (for endosomal escape). For example, fusogenic peptides derived from the influenza virus have been used to enhance endosomal escape of lipid and polymeric carriers for delivery of nucleic acids [86–89].

Another mechanism inspired by viruses and bacteria involves the formation of pores in the endosomal membrane. Pore formation is based on the interplay between membrane tension, which enlarges the pore, and line tension, which closes the pore. Peptides that bind to the edges of pores loosen the internal membrane tension and stabilize pores in the membrane. This process can be induced by cationic amphiphilic peptides such as melittin and cecropin B [90–92]. HIV utilizes the transmembrane protein gp41 and the HIV-1 TAT gene product protein to facilitate endosomal escape, although the exact mechanisms are not well understood. The role of gp41 may be fusogenic or pore forming [83].

Photochemicals, either alone or incorporated into NPs, can be used to disrupt the endosomal membrane upon exposure to light. Photosensitizers, such as meso-tetra (4-sulfonatophenyl) porphine (TPPS4), disulfonated meso-tetraphenylporphine (TPPS2a), aluminum phthalocyanine disulfonate (AlPcS2a), and dendrimerphthalocyanine (Dpc), localize into the membranes of endosomes and lysosomes. After light exposure, these chemicals form short-lived reactive singlet oxygen, which destroys the endosomal/lysosomal membrane and enables the contents of the organelles to be delivered to the cytosol [93, 94].

5.5.2 Cytoplasmic Transport of NPs

The cell cytoplasm is a crowded molecular environment. It contains various macromolecules, the mesh-like cytoskeleton, and many embedded organelles. NPs and large macromolecules have limited diffusion within the cytoplasm. Endogenous proteins, organelles, and vesicles are actively transported along the cytoskeletal network, predominantly via microtubules (MTs) [95, 96]. The transport of endocytotic vesicles is organized by a network of MTs, which radiates from an MT organizing center (MTOC) near the nucleus toward the periphery of the cell. Transport along a MT is mediated by motor proteins such as dynein and kinesin. NPs that incorporate mechanisms to facilitate MT transport (for example, by using ligands with high affinity to dynein, a molecular motor protein responsible for transporting cargo along cytoskeletal microtubules) may be more successful in active transport through the cytoplasm.

5.5.3 Cytoplasmic Targeting of NPs

In some cases, the goal may be to deliver NPs to the cytoplasm itself because the site of action for a given therapeutic (e.g., glucorcorticoids, such as dexamethasone) is located there. Glucocorticoid receptors are located in the cytoplasm; therefore, by delivering the drug at/near its receptor site, a better therapeutic effect may be achieved while minimizing undesirable side effects. Previously, we have demonstrated a sustained antiproliferative effect of dexamethasone-loaded NPs in vascular smooth muscle cells compared with dexamethasone alone, which showed only a transitory effect. Dexamethasone-loaded NPs acted as an intracellular depot, sustaining the antiproliferative effect because of binding of the drug to glucocorticoid receptors present in the cytoplasm [97].

RNA interference (RNAi) also takes place in the cytoplasm. The delivery of siRNAs, for example, has been an area of active research for silencing the expression of genes associated with disease. RNAi oligonucleotides are introduced into cells, cleaved by dicer proteins into siRNAs, and incorporated into the RNA-induced silencing complex (RISC), which then mediates mRNA sequence-specific binding and cleavage to halt transcription of the mRNA into protein [98]. This process occurs within the cytoplasm, although the exact location of the RISC within the cytoplasm is not known. NPs, liposomes, lipoplexes, and polyplexes have been shown to improve the *in vivo* stability, target specificity, and cell and tissue uptake of encapsulated RNAi oligonucleotides [99]. After escaping the endosomes, the nanocarrier must release the siRNA into the cytosol, where it can then interact with the dicer and/or RISC. A better understanding of the location of the RISC within the cytoplasm may lead to further advancements in

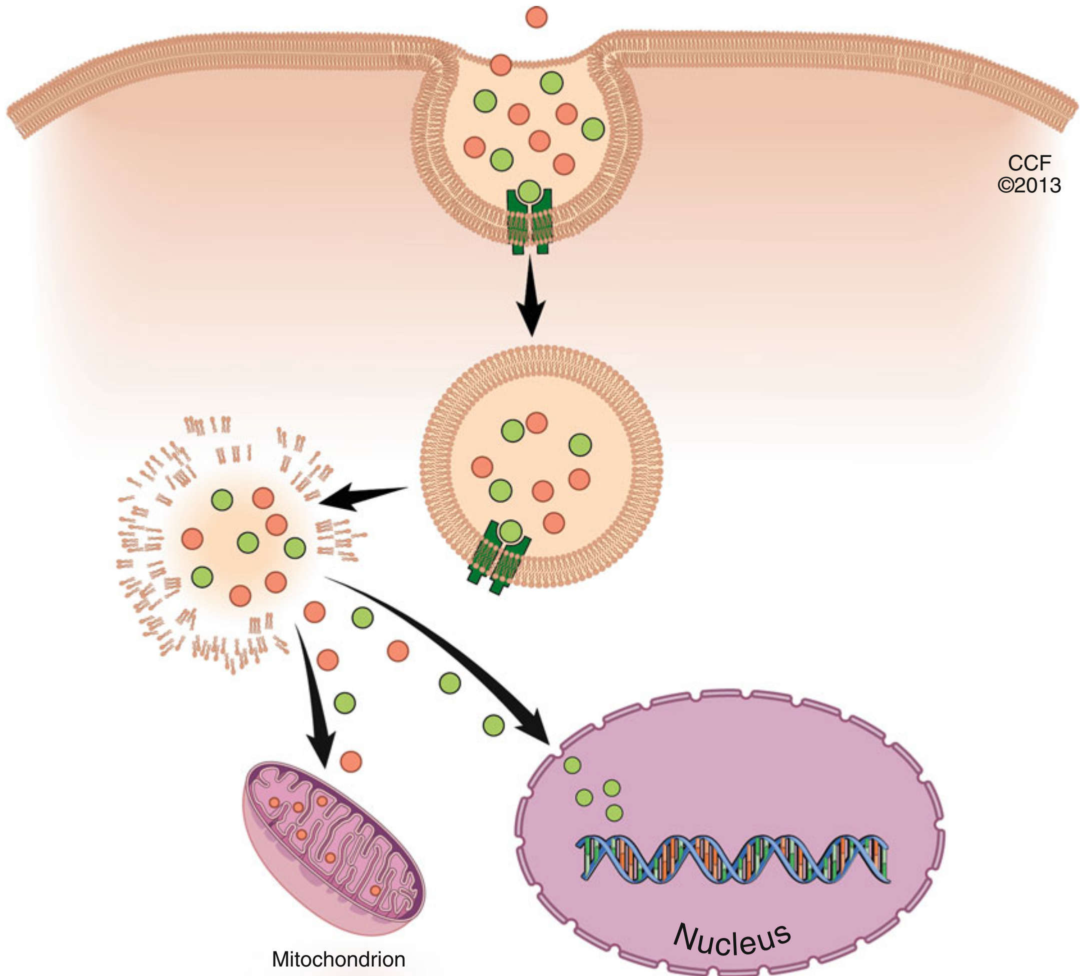

Fig. 5.6 Intracellular trafficking of NPs. Depending on the physical characteristics or presence of a targeting ligand, NPs target different intracellular organelles after endosomal escape into the cytoplasm. NPs that are modified with mitochondriotropic agents localize in the mitochondria, whereas NPs conjugated with nuclear localization signals are transported into the nucleus

intracellular targeting of nanocarriers for RNAi technology.

5.5.4 Nuclear Localization

Nuclear localization of NPs is important for the delivery of therapeutic genes or drugs whose target is the nucleus/DNA. NPs that localize in the perinuclear region have a greater chance of entering the nucleus or having the payload diffuse into the nucleus. Therefore, ideally, NPs must escape from the late endosomes before fusion with the lysosomes. NPs that escape from early endosomes, close to the cell membrane, have to travel the longest distance to get to the nucleus. The nuclear envelope acts as a barrier to the entry of molecules into the nucleus. Entry into the nucleus can occur either by passive or active transport through nuclear pore complexes (Fig. 5.6). Small molecules (<45 kDa) can diffuse freely through the nuclear membrane, whereas larger ones require a nuclear localization signal (NLS) peptide. NLSs are peptides with no general consensus sequence; they are mostly comprised of basic amino acids. Incorporation of NLSs onto NPs has been some-

what successful in improving delivery of NPs to the nucleus [100]. Several NP formulations have demonstrated the ability of NPs to localize in the perinuclear area [101, 102]. This ability may facilitate delivery of the payload to the nucleus, even if the NPs themselves do not actually enter the nucleus. In some cases, NPs have been visualized entering the nucleus [103], which may be more effective for delivery of nuclear targeted drugs/nucleic acids. Nuclear entry of nanocarriers may also be possible during mitosis, when the nuclear envelope temporarily becomes disassembled.

5.5.5 Mitochondria and Other Organelles

Targeting therapeutic agents to the mitochondria is a method that is gaining attention for the treatment of diseases such as diabetes, ischemia-reperfusion injury, cancer, and neurodegenerative diseases. The primary role of mitochondria is energy production for the cell via the electron transport chain, which is crucial for normal cell function and hence body function. Mitochondria also play key roles in regulating cell death and generating reactive oxygen species (ROS) [104]. Mitochondrial dysfunction contributes to a number of diseases, including those mentioned above [105]. As a result, there is increasing interest in drug/therapeutic targeting to the mitochondria for both cytoprotective and cytotoxic applications. Mitochondrial delivery is possible by exploiting some of the inherent characteristics of the organelle. The inner membrane of mitochondria is hydrophobic and anionic, with a high membrane potential (approximately −200 mV) [106]. As a result, lipophilic cations have a tendency to accumulate inside the mitochondrial membrane in response to the membrane potential and are referred to as mitochondriotropics (Fig. 5.6). Triphenylphosphonium (TTP) is one example of a mitochondriotropic moiety that has been incorporated onto the surface of NPs such as liposomes for enhancing mitochondrial accumulation [107, 108]. These systems are under investigation for mitochondrial gene therapy and for anticancer chemotherapy.

In the field of nanocarriers for mitochondrial drug targeting, the majority of approaches use cationic liposomes prepared from trimethylaminoethane carbamoyl cholesterol iodide (TMAEC-Chol) or dequalinium (DQAsomes), as well as branched polyethylenimine (PEI) to delivery peptide-DNA conjugates or micelles. Liposomes are able to fuse with the mitochondria membranes to deliver their cargo. Dequalinium liposomes have been used to bind or entrap drugs such as paclitaxel (a common chemotherapeutic agent) and DNA and transport them to the mitochondria.

Another inherent characteristic of mitochondria that may be used for targeting is their protein import machinery. Although mitochondria have their own DNA, the majority of proteins required by mitochondria are encoded by nuclear DNA. The proteins destined for mitochondria possess a mitochondrial localization signal or mitochondrial targeting signal, which enables them to be delivered to the mitochondria via mitochondrial protein import machinery. It is conceivable that MTS conjugated to nanocarriers could facilitate transport into the mitochondria; however, there are limits to the size of the cargo or carrier that could be delivered via this mechanism.

NP surface chemistry has been shown to affect intracellular localization of NPs. Carboxylate-functionalized polystyrene NPs were found to localize to the mitochondria, whereas plain polystyrene NPs and silica NPs accumulated at reticular and vesicular structures, as well as in the perinuclear region [109].

5.5.6 Methods for Studying Intracellular Tracking of NPs

Evaluating the intracellular location of NPs may be done via a number of microscopic techniques. NPs labeled with fluorescent dye can be visualized intracellularly (in live or fixed cells) using confocal microscopy [110]. Dyes can also be used to label intracellular structures to evaluate where the NPs are in relation to them. For example, LysoTracker Red dye emits red fluorescence in the acidic vesicles of the cell and therefore is

used as a marker of late endolysosomes. This dye process can therefore be a useful tool for distinguishing whether NPs colocalize within the endolysosome or whether they are able to escape the endolysosome into the cytoplasm [110]. Fluorescence recovery after photobleaching has been used to characterize NP transport and dynamics within the cell [111].

5.6 Cytotoxicity of NPs

NP size, charge, surface chemistry, shape, and structure (e.g., porosity, flexibility) can affect the manner in which NPs interact with biological environment and ultimately determine the potential for cytotoxicity. Numerous methods are used to evaluate cytotoxicity both *in vitro* and *in vivo*.

Advantages of the use of *in vitro* systems comprising cell lines for studying cytotoxicity include (1) the ability to determine the primary effects of NPs on target cells in the absence of secondary effects caused by inflammation; (2) the ability to identify primary mechanisms of toxicity in the absence of physiological and compensatory factors that may confound interpretation in whole animals; (3) efficiency, rapidity, and cost-effectiveness; and (4) facilitation/improvements in the design of subsequent whole-animal studies [112]. *In vitro* assays include the following: (1) Assays for cell viability/proliferation (e.g., thiazoylyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide); (2) Mechanistic assays (generation of reactive oxygen species/oxidative stress, apoptosis, necrosis, DNA damage); (3) Microscopic evaluation of intracellular localization (scanning or transmission electron microscopy, video-enhanced differential interference contrast microscopy, AFM, fluorescence spectroscopy, magnetic resonance imaging); (4) Gene expression analysis (high-throughput systems); (5) Hemolysis (evaluates the impact of NPs on human red blood cells by quantifying the release of hemoglobin); and (6) Genotoxicity assays.

Although most cytotoxicity studies for NPs are conducted *in vitro*, they are limited in their ability to recapitulate the complexity of the *in vivo* environment. Acute toxicity studies are conducted to identify the “maximum tolerated dose” and “no observable effect level” of NP dosage. The following parameters are typically monitored in experimental rodent models:

- **Response to administered dose**: Shortly after administration of NPs (<30 min), hematological, cardiac, and neurological responses can occur.
- **Weight change**: This simple and feasible outcome measure is a sensitive indicator of overall animal health, although it is not specific. Further investigation of weight loss is required to determine cause of toxicity and target tissues/organs involved.
- **Clinical observation**: The functioning of organ systems may be evaluated based on clinical changes. For example, cardiovascular system function can be evaluated by the presence of cyanosis of tail, mouth, or footpads; vasodilation can be assessed by redness of skin and vasoconstriction by coldness of body. Respiratory effects manifest as dyspnea (shortness of breath). Gastrointestinal function may be assessed by amount of food consumed and quality of droppings. Imaging procedures, such as ultrasound, X-ray, computed tomography, and magnetic resonance imaging, may also be used to evaluate specific organ toxicity.
- **Mortality**: To ensure humane and ethical use of animals, animals should be euthanized if severe side effects are observed and the animal is not recovering.
- **Clinical pathology**: Analysis of blood and plasma to check blood counts and functioning of liver, kidney, heart, and the endocrine and exocrine systems.
- **Gross necropsy**: This step includes gross examination of each major organ and determination of organ weights, followed by histopathology for signs of toxicity.

Subacute toxicity studies are an extension of acute toxicity evaluation and are generally tailored to detect adverse effects that develop over 4–5 weeks (vs. 2 weeks for acute studies). NPs have distinct pharmacokinetic patterns compared with classic small-molecule drugs. NPs tend to

have longer blood circulation times and tend to localize in the liver and spleen. Understanding the pharmacokinetics of NPs is important to assessing toxicity. Quantification of NPs in organs of the reticuloendothelial system (liver, spleen, bone marrow, lymph nodes, and intestinal Peyer's patches) is of particular importance, due to the tendency of NPs to accumulate in these tissues. *In vivo* tracking of NPs is commonly done using radiolabelling, although other methods such as optical imaging are also used [6, 33, 113–115].

NPs that are not biodegradable, including metal oxides such as gold, carbon, and silica NPs, require longer periods of observation for subchronic (~13 weeks) and chronic toxicity (18–30 months). Such studies typically involve repeated exposures of the NPs under investigation. In addition to the parameters already discussed, ophthalmological examination, cardiac function, neurotoxicology, and immunotoxicology are also evaluated.

The following section describes the impact of NP properties on *in vivo* toxicity. Size and surface charge impact the cytotoxicity of NPs. Amine-terminated poly (amidoamine) dendrimers demonstrated greater *in vivo* toxicity (as determined by maximum tolerated dose) compared with carboxyl- and hydroxyl-terminated poly(amidoamine) dendrimers [116, 117]. The observed toxicity was related to an intravascular coagulation-like condition and hemolysis [118]. In addition, amine-terminated dendrimers were found to accumulate almost entirely in the liver, while carboxyl- and hydroxyl-terminated dendrimers demonstrated greater circulation time and renal clearance. Toxicity was not size dependent; therefore, it was likely related to increased protein opsonization. Size, however, did affect the toxicity of silica NPs (SNPs). For example, 50 nm SNPs were well tolerated *in vivo*; however, 200-nm SNPs with similar surface chemistry were six times less tolerated [116]. In this case, toxicity was determined to be due to embolization in the lungs, which occurred to a greater extent with larger SNPs. These studies indicate that the inherent material properties of the NPs used determine their size-related toxicity. Dendrimers are more flexible nanostructures with hollow spaces that offer less resistance to blood flow compared with silica NPs. This characteristic may also explain why mesoporous SNPs are better tolerated than nonporous SNPs. The response of the biological environment and protein interactions to NPs may vary greatly with the porosity of the NP, since pores allow for different molecular arrangements.

5.7 Concluding Statement

Making generalizations about NP-cell membrane interactions and their potential therapeutic impact or toxicity is difficult because of the use of different NPs, each one with unique features, and variations in cell membrane properties, which differ between tissues and depending on disease conditions. In addition, the same NPs could be used for different applications. It is quite clear that there cannot be a single NP formulation that would work for all applications or in many disease conditions. In this regard, computer simulation and models are being developed that can help in making certain predictions on NP-cell interactions and NP efficacy and potential toxicity. It is also important to correlate the *in vitro* findings to translation *in vivo*. With better understanding and background knowledge on NP-cell interactions, one could more confidently develop a strategy in designing effective NPs for biomedical applications.

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