

Engineered nanomaterial uptake and tissue distribution: from cell to organism

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Abstract: Improved understanding of interactions between nanoparticles and biological systems is needed to develop safety standards and to design new generations of nanomaterials. This article reviews the molecular mechanisms of cellular uptake of engineered nanoparticles, their intracellular fate, and their distribution within an organism. We have reviewed the available literature on the uptake and disposition of engineered nanoparticles. Special emphasis was placed on the analysis of experimental systems and their limitations with respect to their usefulness to predict the *in vivo* situation. The available literature confirms the need to study particle characteristics in an environment that simulates the situation encountered in biological systems. Phenomena such as protein binding and opsonization are of prime importance since they may have a strong impact on cellular internalization, biodistribution, and immunogenicity of nanoparticles *in vitro* and *in vivo*. Extrapolation from *in vitro* results to the *in vivo* situation in the whole organism remains a challenge. However, improved understanding of physicochemical properties of engineered nanoparticles and their influence on biological systems facilitates the design of nanomaterials that are safe, well tolerated, and suitable for diagnostic or therapeutic use in humans.

Keywords: biodistribution, cellular transport, cellular uptake, endocytosis, engineered nanomaterials, nanosafety

Introduction

Engineered nanomaterials (ENMs) are defined as materials composed of particles in an unbound state, or as an aggregate or agglomerate with one or more external dimensions in the size range from 1 nm to 100 nm.¹ Since active cellular uptake and tissue translocation of ENMs have been described for particles larger than 100 nm,^{2,3} we included literature reports on ENMs up to a size of 300 nm. There are many examples of clinical uses of ENMs. The majority of ENMs used as therapeutics on the market and in late clinical studies have diameters above 100 nm.⁴ Small particles with a size of less than 2 nm show passive uptake into erythrocytes.²⁷ However, uptake mechanisms of such very small particles will not be discussed in this review. Due to their small size, ENMs have unique properties (ie, optical, thermal, catalytic, and biological) compared to larger particles.^{5,6} During the last two decades, ENMs with tailored physicochemical properties have emerged in different fields of our daily life. They are used for a variety of applications, such as color pigments, solar cells, and waste water treatment. Furthermore, ENMs are found in consumer products that may be in contact with the human organism, eg, food packaging, shampoos, sunscreens, and toothpastes. Thus, regulatory agencies are faced with new materials for which no

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nano-specific safety standards have been established. Moreover, products containing ENMs are often not declared since formal requirements are lacking.⁵ The ingredients of ENMs tend to be listed as chemicals or micronized substances, and information about the ENMs' content in the product may be missing. Little is known on how ENMs interact with the environment, including animals and human beings.⁷ When used in a physiological environment, ENMs are faced with biological fluids, phospholipid membranes, clearing mechanisms, and harsh intracellular conditions. Due to their distinct physicochemical properties, ENMs interact in a different way with living cells as compared to dissolved molecules. It is a challenge to predict the mechanism of uptake in relation to one specific physicochemical property. Figure 1 highlights the differences between ENMs and small molecules with regard to their physical and chemical properties, cellular uptake mechanisms, intracellular fate, and toxic effects.

Small molecules are defined as compounds with a molecular weight of less than 1,000 Da. It is generally believed that lipophilic molecules below this threshold are able to penetrate cell membranes by passive diffusion. They have the potential to be taken up actively as well as passively

by cells and to overcome cellular barriers within the body including the blood–brain barrier.^{8,9} In contrast, ENMs and macromolecules are mostly unable to diffuse passively into a living cell. They are colloiddally dispersed and therefore require an active transport process for their uptake by target cells.^{10,11} Furthermore, ENMs are characterized by a high surface area to volume ratio as well as different geometries and surface characteristics. Particles of the same material can differ in shape, size, and porosity; whereas a molecule is a well-defined system.¹² The state of dispersion and the variable size and shape of ENMs induces different uptake mechanisms for the same material.

The present review focuses on interactions of ENMs with biological systems on a cellular level (ie, mechanisms of cellular uptake and intracellular accumulation) and on the level of the whole organism (ie, circulation, distribution, and elimination). These interactions are a function of the intrinsic physicochemical properties of ENMs. An additional factor is protein binding. Protein adsorption onto the surface of an ENM leads to the formation of a protein corona and changes properties such as size or surface charge dramatically.^{13–15} We reviewed published experimental procedures since the

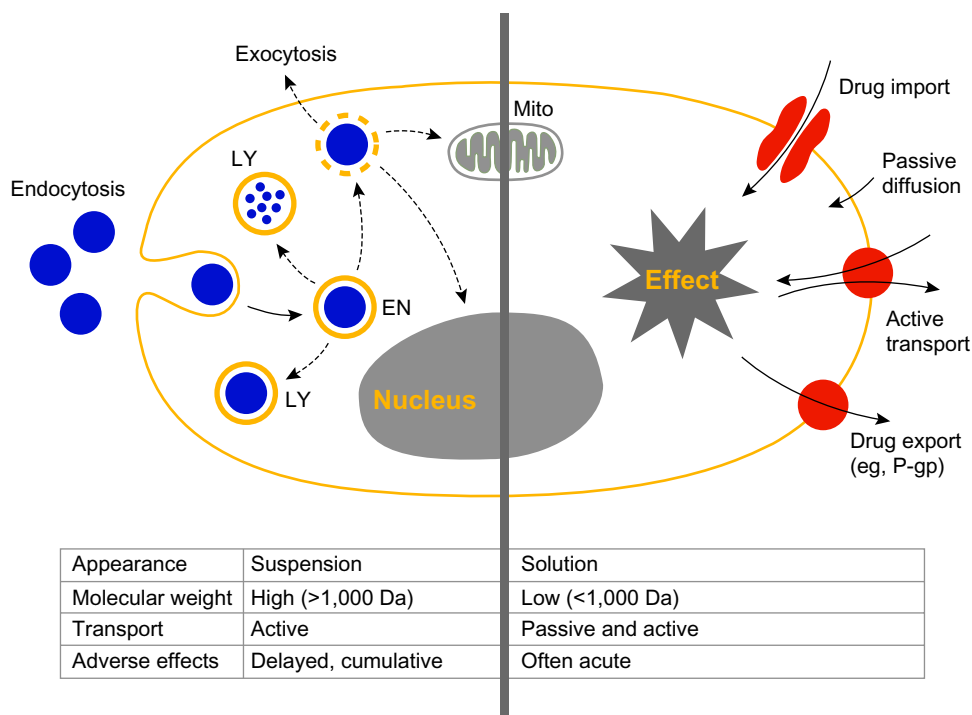


Figure 1 Interactions of cells with small molecules and nanoparticles.

Notes: Schematic representation of a eukaryotic cell and its interaction with nanoparticles (left part of picture) and small molecules (right part of picture). Interactions with nanoparticles are preceded by active cellular uptake leading to intracellular accumulation. Acute effects induced by small molecules are a consequence of both active and passive cell membrane permeation. Endocytosis leads to uptake of particles into endosomes (EN) and lysosomes (LY), followed by lysosomal degradation. Endosomal escape may lead to accumulation of particles in the cytoplasm or within mitochondria (Mito).

Abbreviations: EN, endosomes; LY, lysosomes; Mito, mitochondria; P-gp, P-glycoprotein.

handling of ENMs is often a challenge, leading to statistical variability and artifacts.

General considerations

The state of dispersion of ENMs depends on their surface properties and the medium composition. Uptake studies should be performed with nonagglomerated ENMs. Agglomeration occurs by material interaction (ie, association of ENMs into clusters) or material-protein interaction. The resulting agglomerates sediment according to Stoke's law. The rate of agglomeration should be studied prior to *in vitro* uptake studies in the correspondent medium, for example, with dynamic light scattering (DLS). However, when complex biological media are involved (as *in vivo*), DLS is unsuited for studying agglomeration kinetics since blood components may interfere, fluorescence single particle-tracking (fSPT) may be an alternative method to be used under these conditions.¹⁶

Since the majority of ENM uptake studies use fluorescence as a tracking signal, it is crucial to minimize dye leakage from the ENM.¹⁰ Approaches to test dye leakage are centrifugation, sodiumdodecyl sulfate polyacrylamide gel electrophoresis (PAGE), or fluorescence correlation spectroscopy (FCS).¹⁷⁻¹⁹ Special care should be taken when choosing an appropriate dye since lipophilic dyes may partition from the particulate structure into the lipid membranes of the cell. To circumvent dye leakage, intrinsic fluorescence of nanoparticles is an attractive alternative, as has been described for gold.^{20,21}

To study the entry route of a specific ENM, physical and pharmacological blockers may be used. All methods used to induce a physical or pharmacological block of uptake pathways have certain limitations. Most pharmacological inhibitors are not specific and may influence alternative internalization routes and the actin cytoskeleton.²² Pharmacological inhibitors interfere with vital cell functions and are therefore intrinsically toxic. Experiments have to be designed to prevent toxic effects, such as the complete disruption of all actin-dependent processes by actin-interfering agents. Moreover, compensatory routes of uptake may be activated in target cells upon inhibition of specific transport pathways. Finally, it should be taken into account that different cell lines have different uptake strategies. This impedes extrapolation of experimental results from one cell line to another. A recent review by Iversen et al summarizes the pharmacological blocking strategies and highlights the pitfalls of these reagents.²³ It should be mentioned that

genetically modified cells offer an interesting alternative to pharmacological intervention.²⁴

The influence of plasma protein binding on opsonization

The properties of ENMs have the potential to modulate biological interactions between particles and target cells by different molecular mechanisms.^{6,25} Adsorption of biomolecules to surfaces influences the interactions at the nano-bio interface.²⁶⁻²⁸ In this way, bound proteins and biomolecules form a dynamic protein corona shaping the biological identity of the ENM. The composition of the protein corona varies over time due to continuous protein association and dissociation.^{29,30} Surface properties of ENMs will influence the composition of the protein corona. Hydrophobic ENMs easily adsorb proteins whereas hydrophilic ones are less prone to protein binding.³¹ Therefore, hydrophobic ENMs agglomerate readily and interact with other hydrophobic residues of proteins or peptides (eg, blood or membrane proteins), thus promoting internalization.³⁰ Equally, positively charged ENMs adsorb different sets of proteins on their surface than negatively charged ones, elucidating distinct cellular interactions.³² This influences the mode of cell entry, biodistribution, and biocompatibility of ENMs. Interestingly, ENMs with identical chemical composition but different size may attract different proteins, thereby giving rise to different biological identities.³³ An ENM in blood will have a different biological identity than the same ENM in body fluid and is therefore processed differently. The protein corona, in turn, modifies certain properties of the original ENM, such as surface charge and size. For example, highly positively charged nanoparticles with a positive zeta-potential may change their apparent charge to negative in cell-culture medium.³⁴ These factors should be carefully considered when using ENMs as drug delivery devices, as the targeting ability of functionalized ENMs may be shielded by adsorbed proteins.³⁵

Cellular uptake of nanoparticles

The phospholipid membrane of cells regulates the transport of molecules into the cells, thereby representing a universal barrier protecting fragile intracellular structures from extracellular materials (Figure 2). To enter the cell, polar or charged biomolecules, such as amino acids, nucleosides, or glucose, require active transport systems involving proteins or ion channels. Many macromolecules are actively taken up by cells via endocytosis, which is a vesicular transport mechanism.^{36,37} Because ENMs may exist in the size range

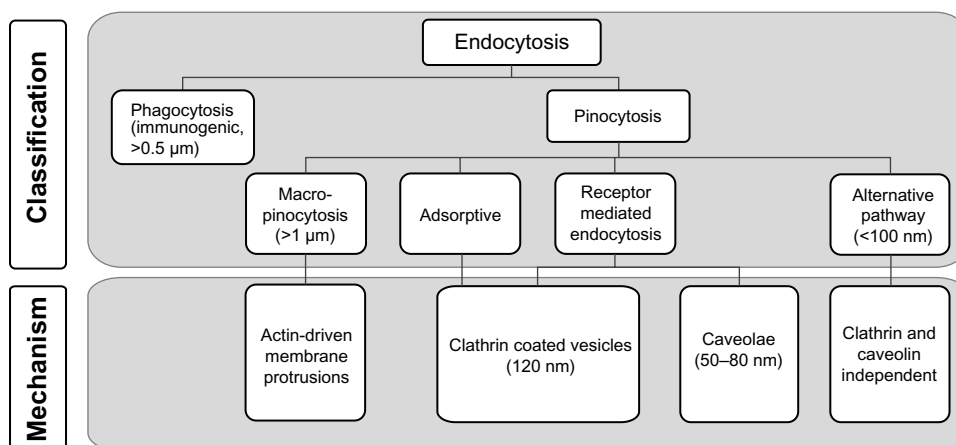


Figure 2 Known pathways of cellular uptake of nanoparticles.

Notes: Uptake of nanoparticles by eukaryotic cells is an active process. Endocytotic pathways include phagocytosis, and pinocytosis.

of proteins, eg, the hydrodynamic radius of an IgG antibody is close to 5 nm,³⁸ they are able to interact with the cellular machinery in a similar way to macromolecules.^{10,25,26} Experimental studies revealed that most ENMs are actively incorporated into the cell via different endocytic pathways (Figure 2) comprising phagocytosis (“cell-eating”) and pinocytosis (“cell-drinking”). The latter pathway can be divided into clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and alternative routes.^{39,40} The resulting transport vesicles differ with respect to the composition of the protein coat of the engulfed vesicle, size of the detached vesicle, and fate of the ingested cargo. ENMs have the potential to access cells by all entry portals depending on their size, shape, chemical composition, and surface modification. As stated above, it remains a challenge to correlate a given ENM property with an uptake route. In the following, we discuss the main cellular entry routes (ie, phagocytosis and pinocytosis) to establish a tentative link between nanoscale characteristics of ENMs and specific mechanisms of cellular uptake.

Phagocytosis

Phagocytosis is performed predominantly by specialized cells of the immune system (ie, macrophages, monocytes, neutrophils, and dendritic cells), to remove particles larger than 500 nm from the organism, mainly through the mode of a receptor-mediated process (Figure 2).^{41,42} Particles are recognized by small proteins (opsonins) including immunoglobulins type G or M, complement fragments (C3, C5), or blood serum proteins such as fibronectin and laminin.⁴³ This process promotes the specific binding of protein-coated particles to internalizing receptors on the cell plasma

membrane, ie, Fc receptors of the immunoglobulin superfamily or complement receptors.⁴¹ Other receptors involved in the uptake of ENMs are the mannose/fucose receptor^{44,45} as well as the scavenger receptor,⁴⁶ where the involvement of the latter one may depend on the cell line used. The receptor-ligand interaction triggers a signal cascade in the target cell resulting in actin assembly and formation of a cell surface extension that zippers around the particle, engulfing it in an intracellular vesicle with a diameter of approximately 0.5 to 1 μm .⁴² These vesicles, or phagosomes, mature by several fission and fusion events with late endosomes and lysosomes, resulting in the formation of phagolysosomes. Internalized particles are subsequently degraded, and the receptors are cycled back to the cell surface. The rate of these successive events depends greatly on the ingested particle and typically lasts from 30 minutes to several hours.⁴⁷ Although phagocytosis is generally thought to be involved in the uptake of larger particles, ie, particles sized >500 nm, recent reports document the phagocytic uptake of considerably smaller particles.^{48–50} The phagocytic uptake route seems to be rather unspecific since it depends on opsonization. ENMs taken up as agglomerate tend to be less easily degraded by the host as they can be detected in macrophages for several months, thus bearing a risk of long-term toxicity.^{51,52}

Pinocytosis

Small particles ranging from a few nanometers to several hundred nanometers are generally taken up by pinocytosis (ie, fluid-phase uptake) occurring in almost all cell types (Figure 2). There are four modes of pinocytosis, ie, macropinocytosis, adsorptive and receptor-mediated endocytosis, and alternative routes of uptake.³⁶ With macropinocytosis, large

amounts of external fluid are taken up nonspecifically. ENMs located near the plasma membrane are thereby coincidentally internalized. During adsorptive pinocytosis, ENMs interact in a nonspecific manner with generic complementary binding sites on the cell surface. In contrast to macropinocytosis and adsorptive pinocytosis, RME is highly specific since it depends on the interaction between a ligand (eg, insulin or transferrin) and its complementary receptor on the surface of a target cell. RME is mediated predominantly either via the clathrin pathway or the caveolin pathway, depending on the specific nanoparticle-receptor interaction.^{23,53} Thus, several distinct pinocytic entry portals exist, depending on the type of interaction with the plasma membrane, size of incorporated vesicles, and type of proteins involved, eg, clathrin, caveolin, or receptors.³⁶ Thereby, the size of endocytic vesicles of approximately 60 nm (caveolae) and 120 nm (clathrin-coated vesicles) imposes limitations with respect to the maximum size of ENMs entering these pathways. Different mechanisms of pinocytic uptake into cells are discussed in more detail below.

Macropinocytosis

During macropinocytosis, ENMs with a size of $>1\ \mu\text{m}$ are taken up nonspecifically. This process is stimulated, for example, by growth factors interacting with receptor tyrosine kinases.⁵⁴ Activation of the signaling cascade results in the formation of actin-driven circular membrane protrusions that collapse onto the membrane and fuse with it. This generates uncoated endocytic vesicles with a size of $1\ \mu\text{m}$. These macropinosomes are of irregular shape and are handled by the endosomal/lysosomal route.³⁶ Macropinocytosis contributes to the internalization of larger ENMs, albeit in a rather unspecific manner and often in conjunction with other entry mechanism.^{11,55} The capacity of this uptake pathway for ENMs is very high, suggesting a possibility for pharmaceutical delivery.⁵⁶

Clathrin-mediated endocytosis

CME is considered to be the “classical” and best characterized route of cellular entry.³⁷ In polarized cells such as endothelial or epithelial cells, transport is initialized at the apical membrane domain.^{36,57} ENMs with a size of 120–150 nm are internalized within clathrin-coated vesicles entering the endosomal/lysosomal trafficking route.^{58,59} The upper size limit reported for particles entering the cell via this pathway is 200 nm.⁵⁵ CME is either adsorptive¹⁴ or receptor-mediated.⁶⁰ Cationic particles or proteins bind nonspecifically to the negatively charged cell surface.^{61–63}

This triggers adsorptive CME. In contrast, the receptor-mediated process is highly selective and specific. Receptor ligands being internalized by this pathway include low-density lipoprotein (LDL), transferrin, growth factors, and insulin.¹⁴

Caveolin-mediated endocytosis

CvME is the most prominent clathrin-independent uptake mechanism. This pathway is most pronounced in endothelial cells on the basolateral side, where it is an important uptake route for ENMs.^{36,64} The caveola is a small, flask-like structure, with a diameter of about 50–80 nm that is coated with caveolin-1.⁶⁵ These vesicles invaginate with the help of dynamin from hydrophobic membrane domains, which are rich in cholesterol and glycosphingolipids.⁶⁶ Caveolae are stable plasma membrane-associated structures.^{67,68} However, they can be induced to bud off by interaction with pathogens such as SV40 virus,⁶⁹ cholera toxin subunit B, or Shiga toxin.⁷⁰ With respect to ENMs, small particles seem to be transported more efficiently. For example, uptake of nanoparticles of 20 and 40 nm in size was demonstrated to be 5–10 times faster than that of nanoparticles sized 100 nm.^{64,71} Larger particles ($>500\ \text{nm}$) appear to be taken up only in exceptional cases.⁵⁵ However, it is possible that ENM associating with the membrane may cover enough surface and colocalize by chance with certain markers like the caveolar marker. This does not mean that the uptake is actively dependent on caveolae.

The intracellular trafficking routes of caveolae are discussed controversially. While nonacidic, nondigestive pathways are favored, an additional link between caveolae and lysosomal routes for degradation cannot be excluded.^{65,70,72} In addition, the so-called caveosomes (previously supposed to represent a special type of caveolar endosome) were shown to be an artifact created by overexpression of caveolin or caveolin mutants.⁷³ Pathogens escape from normal degradation in lysosomes and are directly transported to the Golgi and/or endoplasmic reticulum.⁷⁴ Thus, pathogens and ENMs can bypass lysosomal degradation.⁷⁵

Alternative routes of uptake

More recently, several endocytic routes that do not fit into the categories described above have emerged. Many of them are clathrin- and caveolin-independent. These routes depend on specific regulation by proteins such as Ras homolog family member A, ADP-ribosylation factor 6, or the cell division control protein 42 homolog, Cdc42.^{37,68} Although these mechanisms are still poorly understood, available data

suggest that particles larger than 100 nm are internalized via these routes.⁶⁶

Characteristics of ENMs influencing cellular uptake

The size of ENMs is not the only criterion that predicts the mechanism of ENM uptake. Nonetheless, trends based on particle size exist and are summarized in Figure 2. Nanoparticles with a diameter of 50 nm are more efficiently internalized by cells than smaller (about 15–30 nm) or larger (about 70–240 nm) particles.^{58,76} Nanoparticles with a diameter of 30–50 nm efficiently recruit and interact with membrane receptors and are subsequently taken up by receptor-mediated endocytosis.⁷⁷

Besides size, ENM shape is an important factor. Spherical ENMs are taken up much faster and more efficiently than rod-shaped ENMs, presumably due to the longer membrane wrapping time required for the longer rod-shaped particles.^{78,79} This notion is supported by *in vivo* experiments in rodents, where intravenous (IV)-injected elongated polymer micelles (filomicelles) remained in the circulation 10 times longer than spherical ENMs.⁸⁰ However, controversial findings were obtained using different materials. For example, Gratton et al⁷¹ investigated hydrogel particle uptake into HeLa cells. The highest internalization rate was found for particles with an aspect ratio of three. ENMs seemed to use all internalization routes simultaneously.⁷² Recent findings suggest that silica rods with an aspect ratio of 2.1 to 2.5 are taken up to a higher extent into HeLa cells than their spherical counterparts. In addition, uptake of rods with higher aspect ratios was marginal.⁸¹ Tissue macrophages struggle to incorporate long and rigid fibers into phagosomes, thereby releasing harmful oxygen radicals and hydrolytic enzymes, causing chronic inflammation.⁸² Similarly, the needle-like structure of carbon nanotubes may penetrate biological membranes, inflicting mechanical damage. These controversial results suggest that additional factors promote cellular uptake besides ENM size and shape.

Surface functionalities (eg, surface charge and functional groups) mediate interactions between ENMs and the cell surface. Positively charged particles interact strongly with the slightly anionic plasma membrane. They are taken up more readily⁸³ or may disrupt plasma membrane integrity.⁸⁴ Positively charged ENMs are taken up via adsorptive mediated pinocytosis, whereas negatively charged ENMs use alternative uptake routes.⁸⁵ Nonionic particles tend not to interact with the cell membrane, as demonstrated for the polymer polyethylene glycol (PEG).⁸⁶ Nanoparticles can be

functionalized with a plethora of ligands such as antibodies, peptides, or sugars. Their density, spatial distribution, and molecular weight plays an important role in determining the fate of ENMs in biological systems.⁸⁷ In addition, the chain length of chemical linkers like PEG, which are used to attach ligands to the surface of nanoparticles, may affect delivery to target cells.⁸⁸

Finally, specific cell types may interact with identical ENMs differently.^{23,89} It has been shown, for example, that macropinocytosis or phagocytosis is absent in brain capillary endothelial cells.⁸ Red blood cells are known to be incapable of endocytosis. Furthermore, recent studies revealed an influence of the cell cycle phase on the cell's capacity to take up ENMs.⁹⁰

Intracellular fate and endosomal escape

Once ENMs have been taken up by target cells, they are directed to the endosomal/lysosomal pathway in most instances. The intracellular vesicles either gradually mature (acidify) to late endosomes through multiple fission and fusion events, or they are recycled back to the cellular surface as trafficking endosomes (Figure 1).⁹¹ ENMs entrapped in late endosomes are likely to proceed to lysosomes where they are degraded. These compartments harbor proteases, hydrolases, and other enzymes promoting ENM degradation.⁹² However, some ENMs (in particular positively charged, basic nanoparticles) are capable of escaping the endosome. This phenomenon has previously been described as the “proton sponge effect”.^{25,93} Osmotic swelling of the organelles leads to their rupture, as shown in the case of polyethylene imine.^{93,94} To implement drug delivery strategies, endosomal/lysosomal escape can be induced actively. pH-sensitive fusigenic liposomes, for example, contain synthetic sterols and phospholipids, which undergo phase transition upon protonation at low pH. This results in the conversion of the hexagonal to lamellar structure of the liposomal membrane, thus disrupting the endosomal/lysosomal membrane.^{95–97} Similar effects can be induced using pH-sensitive fusigenic peptides (eg, amphiphilic peptides with repetitive GALA sequences) in combination with cationic liposomes.⁹⁸ Other ENMs (eg, certain types of carbon nanotubes) penetrate the vesicle (or cell) membrane directly and enter the cytosol.⁹⁹ Once in the cytosol, ENMs may induce the production of reactive oxygen species and inflict oxidative stress.¹⁰⁰ In addition, potentially toxic interactions with other cellular organelles, such as mitochondria and the cell nucleus, may occur.² Effects that may be harmful for a healthy cell are

desired in tumor cells, where an endosomal escape is needed to deliver a specific nanoparticulate drug to its intracellular compartment of action.

Methods to determine nanoparticle uptake

Widely used methods to study cellular uptake of ENMs are flow cytometry and microscopy. While flow cytometry allows for an efficient, fast, and quantitative assessment of particle uptake, microscopy provides qualitative insight into the subcellular localization of particles by analyzing small sample volumes. Flow cytometry as a quantitative approach to measure uptake cannot distinguish between externally attached and fully internalized ENM. Interaction with the cell surface can be studied experimentally if cellular uptake is inhibited. For example, endocytotic pathways can be blocked using pharmacological inhibitors.^{22,101} Alternatively, cells can be incubated at lowered temperatures to interrupt endocytic processing of particles. Temperatures around 20°C prevent progression of particles from early to late endosomes.¹⁰² Further temperature lowering to 4°C, for example, blocks all energy-dependent processes.¹⁰³ Consequently, signals from fluorescent ENMs located at the surface of cells or within specific intracellular vesicles or organelles can, for example, be detected quantitatively by flow cytometry or qualitatively by confocal scanning laser microscopy.^{104,105} Intracellular localization of particles can be further confirmed using the quenching agent, trypan blue. This dye quenches signals from fluorescent dyes such as fluorescein isothiocyanate. Since trypan blue does not penetrate the membrane, only extracellular signals of free or surface-bound dye molecules are quenched.¹⁰⁶

Another quantitative approach is induced coupled plasma mass spectroscopy (ICP-MS). ICP-MS is a powerful tool for intracellular quantification of electron-dense materials and is a sensitive (ie, nanogram range) method for detecting elements with the exception of H, C, O, N, F, S, and inert gases. Due to this limitation, “soft” nanoparticles, such as liposomes, polymers, or dendrimers, are not detected. Additionally, ICP-MS is not able to distinguish between dissolved ions and nanoparticles. However, loading nanoparticles with a heavy metal may make the use of ICP-MS possible in such cases, as long as the physicochemical properties of the particles are not changed by the loading procedure.¹⁰⁷

Alternatively, confocal microscopy provides information on the three-dimensional structure of objects. Fluorescence colocalization studies give insight into intracellular trafficking after fixation of cells or by live-cell imaging. The latter

technique relies on highly photostable fluorophores but avoids artifacts introduced by fixation reagents such as paraformaldehyde.¹⁰⁸ Subcellular fractionation can give additional insight into partitioning of ENMs inside the cell. Transmission electron microscopy is used to confirm subcellular particle localization with high resolution. This method allows quantitative assessments, but the procedure is time-consuming.^{109,110}

It is advisable to use transmission electron microscopy in combination with energy-dispersive X-ray spectroscopy (EDX) to confirm the presence of nanoparticles.¹¹¹ Brandenberger et al studied intracellular particle distribution using quantum dots as reference material.¹¹² In this study, EDX was used to confirm the identity of quantum dots based on X-ray emission spectra of the elements Cd and S. This method seems to be particularly useful for identifying very small (5–10 nm) particles.¹¹³

The biological fate of ENMs

In previous sections, we discussed interactions of ENMs with biological systems on a cellular level. This section focuses on *in vivo* kinetic properties of ENMs, addressing processes related to circulation, distribution, degradation, and excretion. There are different routes of administration including pulmonary,¹¹⁴ dermal,^{115,116} oral,^{117,118} and IV routes.¹¹⁹ However, this review will focus only on the IV route in healthy subjects. Figure 3 illustrates technical hurdles, challenges, and the different steps taken by ENMs before and during interaction with the living organism.

Circulation of ENMs

The state of dispersion in both the injected solution and the blood stream has to be defined, since agglomerates or precipitated material in the syringe may lead to dose variability (Figure 3A). Advanced pharmaceutical technologies are needed to stabilize nanosuspensions during storage and administration.¹²⁰ Size is an important parameter regarding circulation and distribution within the organism. In the blood stream, agglomerates may cause embolism with a potentially fatal outcome. Agglomerated particles have a tendency to accumulate after IV administration within the lung since venous blood is directed from the right heart ventricle to this organ. *In vivo* studies in the rat using polystyrene particles demonstrated passive accumulation in the lung for particles with a size exceeding a threshold of 10 μm .¹²¹ Thus, findings of lung targeting of ENMs may be indicative of “accidental” trapping of agglomerates.¹²² As mentioned above, phagocytosis of ENMs is an additional

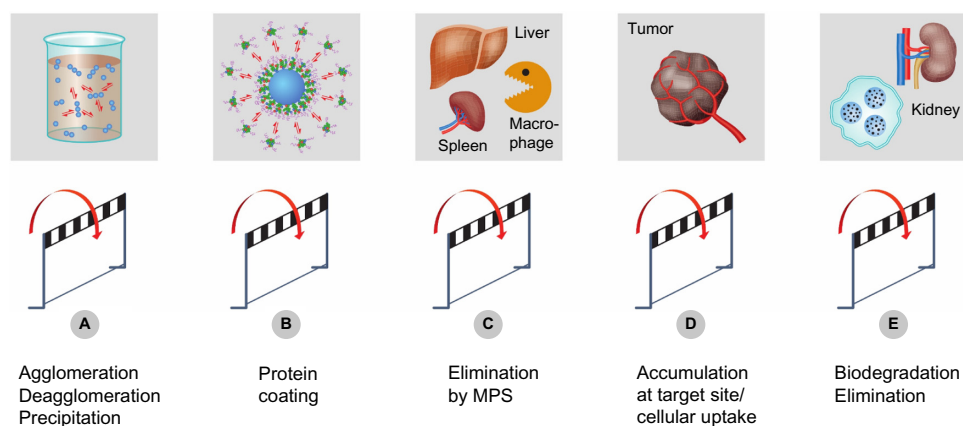


Figure 3 Experimental challenges and hurdles.

Notes: Specific physicochemical properties of ENMs may lead to technical challenges and artifacts in experimental systems. Particle agglomeration (A) reduces dosing accuracy or may lead to embolism after IV injection. Plasma-protein binding and opsonization of nanoparticles (B) may trigger a humoral immune response. Interaction of nanoparticles with cells of the MPS leads to accelerated plasma clearance (C). Accumulation of particles at a defined target site (D) might be impeded by their premature degradation and elimination (E).

Abbreviations: ENMs, engineered nanomaterials; IV, intravenous; MPS, mononuclear phagocytic system.

factor limiting prolonged circulation of particles bigger than $0.5\ \mu\text{m}$ (Figure 2). On the other hand, small ENMs ($<5\ \text{nm}$ to $10\ \text{nm}$) are cleared by kidney glomerular filtration.^{123,124}

Opsonization and immunologic responses

When injected into the blood stream, foreign materials encounter different blood constituents such as red blood cells, white blood cells, platelets, and a variety of proteins. ENMs are known to interact with both proteins²⁶ and cells.¹²⁵ Protein binding and opsonization are processes that change the surface properties of ENMs (Figure 3B). ENMs used for imaging or as a drug delivery system should be assessed with regard to their potential to cause hemolysis, thrombocyte aggregation, and complement activation.^{47,126} Hemolysis has been described for rigid materials such as silica nanoparticles¹²⁷ or for soft nanoparticles such as liposomes.¹²⁸ Due to protein binding, these measurements should be done in presence of plasma proteins.¹²⁹

Protein coating of an ENM is a highly dynamic process, starting immediately upon IV injection.¹³⁰ The resulting protein corona is complex and varies depending on the size,¹³ hydrophobicity/surface charge,³⁰ and shape¹³¹ of the particles. In the first instance, readily available proteins such as albumin are adsorbed onto the ENM surface, but may be replaced by other proteins (eg, lipoproteins or opsonins) over time, depending on the surface structure of the ENMs. Prediction of the extent of protein binding to ENM remains a challenge. Recent findings indicate that uncharged ENMs are opsonized more slowly than charged ENMs. The size of ENMs influences the binding of opsonins to spherical particles. Due to the higher curvature, smaller ENMs ($<20\ \text{nm}$) will attract

fewer opsonins than bigger ENMs ($>200\ \text{nm}$). This explains why agglomerates are cleared by the mononuclear phagocytic system (MPS) more easily. The same is true for hydrophobic ENMs.¹³² Recent studies revealed a strong shape dependency in relation with MPS recognition. Duan and Li¹³³ stated that an ellipsoidal ENM can be attacked by a macrophage in two different ways: when the macrophage attaches to the pointed end, the ENM will be internalized; however, when it attaches on the flat surface of the ellipsoid, it just spreads on the ENM and prolongs its circulation. This observation is in agreement with the findings of Arnida et al who found that gold nanorods were less recognized by the MPS compared to their spherical counterparts.¹³⁴

The question arises as to how ENMs can be designed to specifically adsorb certain proteins or avoid their adsorption. The best known strategy to diminish protein adsorption is by masking the particle surface with PEG. This hydrophilic, biocompatible, and nontoxic polymer is used to minimize interactions of macromolecules, eg, cytokines¹³⁵ and nanoparticles, ie, liposomal carriers¹⁰¹ with phagocytic cells of the immune system. The protective properties of PEG as a function of PEG chain length and PEG surface density were reviewed by Li and Huang.¹³⁶ Nevertheless, PEG is not able to fully prevent protein adsorption or opsonization. For example, for $5\ \text{nm}$ gold nanoparticles, the protective effect of PEG diminishes within hours.¹³⁷ In addition, PEG may elicit an immunological response resulting in an accelerated blood clearance of ENMs. It has been observed that repeatedly injected PEGylated liposomal formulations are markedly less efficacious, probably because of anti-PEG antibody formation.¹³⁸ As an alternative to PEGylation, ENMs can be

coated with amino acids such as lysine or cysteine. Such a mixed-charge monolayer-coating prevented protein adsorption in fetal bovine serum to 5 nm gold nanoparticles.¹³⁹ In summary, a hydrophilic coating (eg, PEG) and a neutral charge (expressed as the zeta potential in the correspondent medium) can minimize particle recognition by the immune system. Targeting strategies are often implemented using PEG-coated ENMs with surface ligands (such as folic acid, cell adhesion molecules, or transferrin⁴²) in order to enhance cellular uptake into target tissues and to avoid exposure of healthy cells. By this approach, it is crucial to maintain the stealth properties of the PEG, despite the bound ligands, to avoid interactions with cells of the immune system.¹⁰¹

Distribution

ENMs are distributed throughout the body via the blood stream and extravasate this transport system according to their size. Extravasation of ENMs is restricted to specific tissues since the presence of tight junctions prevents ENMs larger than 2 nm to leave the circulation.¹⁴⁰ The fenestrations of blood vessels vary from organ to organ and can have different ranges in different species.¹⁴⁰ Moreover, the state of disease is changing the fenestration size,¹⁴¹ with typical size for tumors ranging from 200–780 nm in mice.¹⁴² ENMs with sizes ranging from 150–300 nm are mainly found in the liver and spleen, whereas smaller counterparts extravasate into the bone marrow. ENMs are cleared from the circulation in different organs. As depicted in Figure 3C, they are often trapped in the liver and spleen as these organs host the largest concentration of tissue resident macrophages (ie, MPS cells such as hepatic Kupffer cells). Glomerular filtration eliminates ENMs with a hydrodynamic diameter of <5 nm to 10 nm. Nonetheless, the relationship between the physico-chemical properties and pharmacokinetic behavior of ENMs is poorly understood. Interpretation of experimental data is difficult since a plethora of different materials, excipients, and animal species are used. However, trends were identified for selected materials such as gold nanoparticles of different sizes and surface characteristics.^{143,144} Increased liver accumulation was found for particles with a size of ≥ 5 nm as compared to particles with a size of 1.4 nm. Particle size-dependent accumulation was found in no other organ than the liver. However, elevated levels of charged (positive and negative) gold nanoparticles were found in the spleen. In another study, PEG-coated gold nanoparticles with a size of 20–80 nm were investigated.¹⁴⁵ The length of the PEG chains was 2,000–5,000 Da. PEGylated gold nanoparticles were not recognized by macrophages. The PEG-5,000 coated

gold particles with a size of 20 nm accumulated in solid tumors of experimental animals to an extent of 6.5% of the injected dose. Most of the remaining particles accumulated in other organs, mainly liver and spleen. Xie et al investigated the influence of the size of silica nanoparticles on their biodistribution.¹⁴⁶ After 30 days, they found silica particles trapped in the lungs, liver, and spleen and observed signs of liver injury. Compared to small silica particles (20 nm), larger particles (80 nm) were cleared faster from the body. A recent study investigated the difference in biodistribution of solid silica nanoparticles, mesoporous silica nanoparticles, and rod-shaped silica nanoparticles.¹⁴⁷ All particle types had a positively charged surface and showed extensive distribution to liver and spleen. Thus, most particles were recognized and eliminated by phagocytic macrophages. Porous particles with an aspect ratio of eight preferentially accumulated in the lung, whereas the nonporous particles were less prevalent in the lung. Amine modified silica nanoparticles reduced the affinity to lungs and kidneys. Silica nanoparticles were degraded and excreted via the hepatobiliary and renal routes. It has been reported that elongated fibers such as filomicelles⁸⁰ or gold nanorods¹³⁴ display a longer circulation time since they are able to align with the blood flow. Compared to their spherical counterparts, rods are less preferentially taken up by macrophages, which reduce accumulation in MPS organs like the liver and spleen. Furthermore, gold nanorods had a higher accumulation in tumor tissue compared to spherical gold nanoparticles.¹³⁴

ENMs can be designed in a way to promote or avoid interactions with specific tissues or organs. The topic of active drug targeting was recently reviewed by Moghimi et al.¹⁴⁸ With such targeting strategies, ENMs can be designed and used for diagnostic imaging purposes or to deliver drugs to diseased tissues such as solid tumors (Figure 3D). The passive accumulation in the tumor is due to the enhanced permeability and retention effect, which is present in some tumors and dictates the maximum size for ENMs to extravasate into tumor tissue. This phenomenon is discussed in more detail by Jain.¹⁴⁹

Degradation and excretion

The term “metabolism” as defined in classical pharmacokinetics is not suitable for ENMs. In this section, the term “degradation” will be used instead to collectively cover multiple processes such as erosion, deagglomeration, disintegration, dissolution, or chemical degradation of particles. Available excretion and degradation studies solely included single administration of ENMs at a certain concentration.

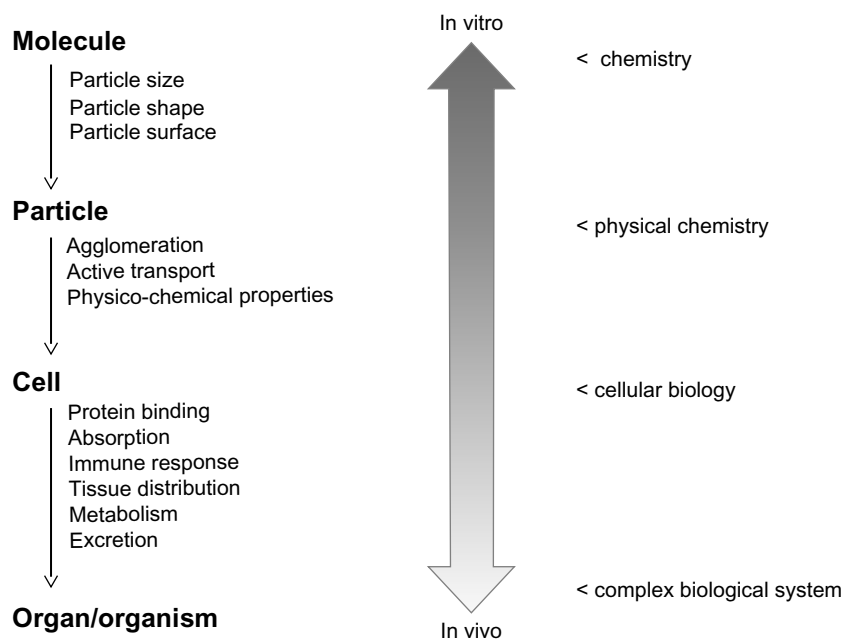


Figure 4 Extrapolation from in vitro data to the in vivo situation.

Notes: In vitro experimental systems can be used to characterize nanoparticles with respect to their chemical composition and physicochemical properties. Cell-culture-based experimental systems can be used to study molecular mechanisms of cellular uptake and intracellular processing of particles. However, additional information is needed to address questions related to the in vivo behavior of nanomaterials and their interaction with complex biological systems. In particular, the prediction of pharmacokinetic parameters remains a challenge.

Accumulation effects after multiple dosing and bioaccumulation have only been studied in zebra fish.¹⁵⁰ Chemical reactivity and composition of the shell and core materials play an important role in degradation. ENMs known to “safely” degrade are porous silica nanoparticles²⁰ and iron oxide particles.¹⁵¹ Degradation of silica nanoparticles leads to the formation of silicic acid, which is excreted via feces and urine.¹⁵² Due to the high specific surface area of the mesoporous material, hydrolysis of the silica network is a fast process.¹⁵³ Metal oxides including iron oxides are transformed by metallothionein that is abundantly expressed in liver and kidney.¹⁵⁴ Levy et al used two different methods to trace biodegradation of super paramagnetic iron oxide nanoparticles (SPIONs).¹⁵⁵ Upon degradation, these iron species lose their paramagnetic behavior and are transformed to ferritin. Thus, intact particles can be identified and traced due to their magnetic properties. However, degradation is a slow process. After two months, paramagnetic iron was still present in macrophages. Over prolonged time, the storage form of iron (ie, nonparamagnetic species) was more prevalent. The authors hypothesized that degradation of SPIONs took place in the acidic lysosomal compartment. It remains to be elucidated if these mechanisms of degradation might apply to other nanomaterials including other metal oxide materials with poor solubility. ENMs, like polymeric particles, have been shown to

degrade by hydrolysis or enzymatic digestion in vitro.¹⁵⁶ Similar effects were observed in vivo.¹⁵⁷

Materials with poor solubility may remain in the organism over several weeks to months. When considering the use of a specific ENM as a drug delivery tool, its biodegradation and excretion pathways have to be known. Upon multiple dosing, ENMs may accumulate in MPS organs and cause severe damage. For example, Ye et al have studied long-term effects of quantum dots containing Cd-Se in rhesus monkeys.¹⁵⁸ In vivo, acute toxicity of these nanoparticles was very low. However, chemical analysis after 90 days revealed that more than 90% of the injected Cd dose remained in the animals’ organs. In view of the limited availability of data, much more work needs to be done in the field of nanosafety.

Conclusion

ENMs have emerged in different fields of our daily life. However, their interaction with biological systems and their biological fate remain incompletely understood. It is therefore important to elucidate molecular mechanisms involved in cellular binding, uptake, and processing of ENMs. This knowledge is needed to design novel pharmaceutical applications for ENMs such as, for example, drug delivery and drug targeting strategies. By the same token, optimized ENM design may help to avoid unwanted interactions and toxicity, thereby making human use of novel materials possible.

The interaction of ENMs with complex biological systems, such as the human body, is still poorly understood. In vitro characterization of ENMs may help to obtain a mechanistic insight into their behavior in a biological environment (Figure 4). This includes information on chemical properties and reactivity as well as particle dynamics in biological fluids. Studies have shown that protein binding may alter the properties of ENM surfaces influencing cellular binding and uptake. Interactions with the immune system depend on ENM size, geometry, and surface charge. However, this information cannot be easily extrapolated to any given cell line or even to another ENM with the same surface properties but different core materials. We propose to combine in vitro systems with ex vivo models such as lung models,¹⁵⁹ cell coculture systems, chicken egg models harboring xenografted tumors,⁵⁰ and placenta models.³ These tools will be instrumental when designing nanomaterials with favorable pharmacokinetic properties and low intrinsic toxicity.¹⁶⁰ In any case, ENMs should be designed to be biocompatible and biodegradable to prevent their accumulation in the human body and limit their long-term toxic effects upon chronic exposure. Combining this knowledge about ENMs with smart drug delivery and drug targeting strategies will lead to innovative diagnostic and therapeutic applications.

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Disclosure

The authors report no conflicts of interest in this work.

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