

The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems

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Abstract

An understanding of the interactions between nanoparticles and biological systems is of significant interest. Studies aimed at correlating the properties of nanomaterials such as size, shape, chemical functionality, surface charge, and composition with biomolecular signaling, biological kinetics, transportation, and toxicity in both cell culture and animal experiments are under way. These fundamental studies will provide a foundation for engineering the next generation of nanoscale devices. Here, we provide rationales for these studies, review the current progress in studies of the interactions of nanomaterials with biological systems, and provide a perspective on the long-term implications of these findings.

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INTRODUCTION

Nanotechnology is defined as the “the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale” (<100 nm) (1, p. 9). Over the past three decades, nanotechnology has emerged as a promising strategy to resolve the technological impasses incurred in various branches of science. Nanotechnology research has focused on understanding the correlation between the optical, electrical, and magnetic properties of nanomaterials with respect to their size, shape, and surface chemistry (2, 3). These studies have provided a solid foundation for engineering nanotechnology-based electronic, computer, and biomedical devices. A recent trend in nanotechnology has been to investigate the interactions of nanomaterials with biological systems, known as nano-bio interactions. Experimentally, researchers design a nanoparticle series where all parameters are kept constant with the exception of one in the experimental framework (**Figure 1**). These nanoparticles are

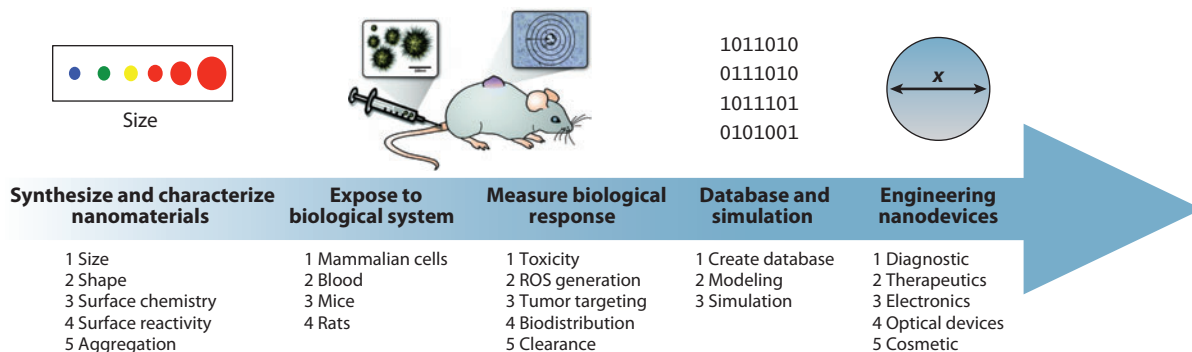


Figure 1

Overview of nano-bio interactions and their impact on the nanoengineering process. Typically, nanoparticles with a single or combination of known variable(s) (e.g., size, or size and surface chemistry) are exposed to a biological system (e.g., mice with tumors), and a biological response is measured (e.g., blood half-life). By systematically examining one nanostructure parameter at a time, researchers are able to draw correlations between the nanoparticle design and a specific biological response. These data can then be used to populate a database to develop predictive software on how nanoparticles behave in that biological system. Such data could guide the engineering of the next generation of nanoscale devices. Listed are the most prevalent nanoparticle parameters, biological systems, biological responses studied, and potential applications. Abbreviation: ROS, reactive oxygen species. Image used with permission (38).

then exposed to plants, animals, cells, or tissues, and a biological outcome, such as toxicity, is measured. By analyzing different nanoparticles systematically, one is then able to determine if a single or combination of nanoparticle parameters is responsible for a specific biological response. The significance of these studies is the identification and establishment of design rules that govern the engineering of nanodevices. In this review, we describe the rationale for these studies and provide an overview of the current state of knowledge on nano-bio interactions. In the final section of this article, we demonstrate how nano-bio studies have influenced the design of nanoparticles in the past decade and now require further studies to enable researchers to build a database that can be used to predict the behavior of nanomaterials in complex biological systems.

RATIONALE

Molecules, viruses, bacteria, and other biological structures have evolved into precise sizes, shapes, and chemistries to mediate interactions and functions. The F_{AB} branches of an antibody recognize complementary antigen targets and bind to them using noncovalent forces. Viruses are icosahedral, bullet-shaped, or rod-shaped or they can have asymmetric morphologies. These geometries may dictate their ability to infect specific cell types and may alter their residence time inside the cell. Proteins assemble in complex units, such as ribosomes, to regulate cell viability and function. All these biological molecules and structures are in the nanometer-size range. Although we have not fully elucidated how the physicochemical properties of naturally occurring nanosized complexes and structures influence their function, it is clear that these biological structures follow design rules (4). It will be important to understand how the physicochemical properties of nanostructures relate to biological interactions and functions in order to adequately materialize the potential of nanotechnology.

If we want to engineer nanostructures that are capable of navigating the body, infecting and transforming cells, or detecting and repairing diseased cells, we have to develop an understanding of how the physicochemical properties of a synthetic nanoparticle interact with biological systems. We are able to engineer nanostructures with a variety of sizes, shapes, and chemistries; we can also synthesize biological molecules such as peptides and oligonucleotides. In our current research paradigm, nanoparticles are initially synthesized, surface modified, and finally exposed to biological environments or incorporated into devices. Early studies on nanomaterials did not consider the final application to guide the design. Instead, the focus was predominantly on engineering materials with specific physical or chemical properties. For example, when developing nanostructures for tumor diagnosis, researchers considered the optical and magnetic properties in improving the detection sensitivity. However, the delivery efficiency of these materials plays a dominant role in determining signal intensity. By identifying how size, shape, and chemistry influence the delivery process, one is then able to redesign the nanoparticles so that they accumulate maximally in the tumor. The fundamental studies on nano-bio interactions will enable the parameterization of the nanoengineering process by creating specific design rules. This would enable researchers to streamline the construction of complex nanostructures that ensure the highest possible delivery efficiency while maximizing signal strength.

STATE OF KNOWLEDGE

In Vitro Studies

A prototypical nanoparticle is produced by chemical synthesis; then coated with polymers, drugs, fluorophores, peptides, proteins, or oligonucleotides; and eventually administered into cell cultures

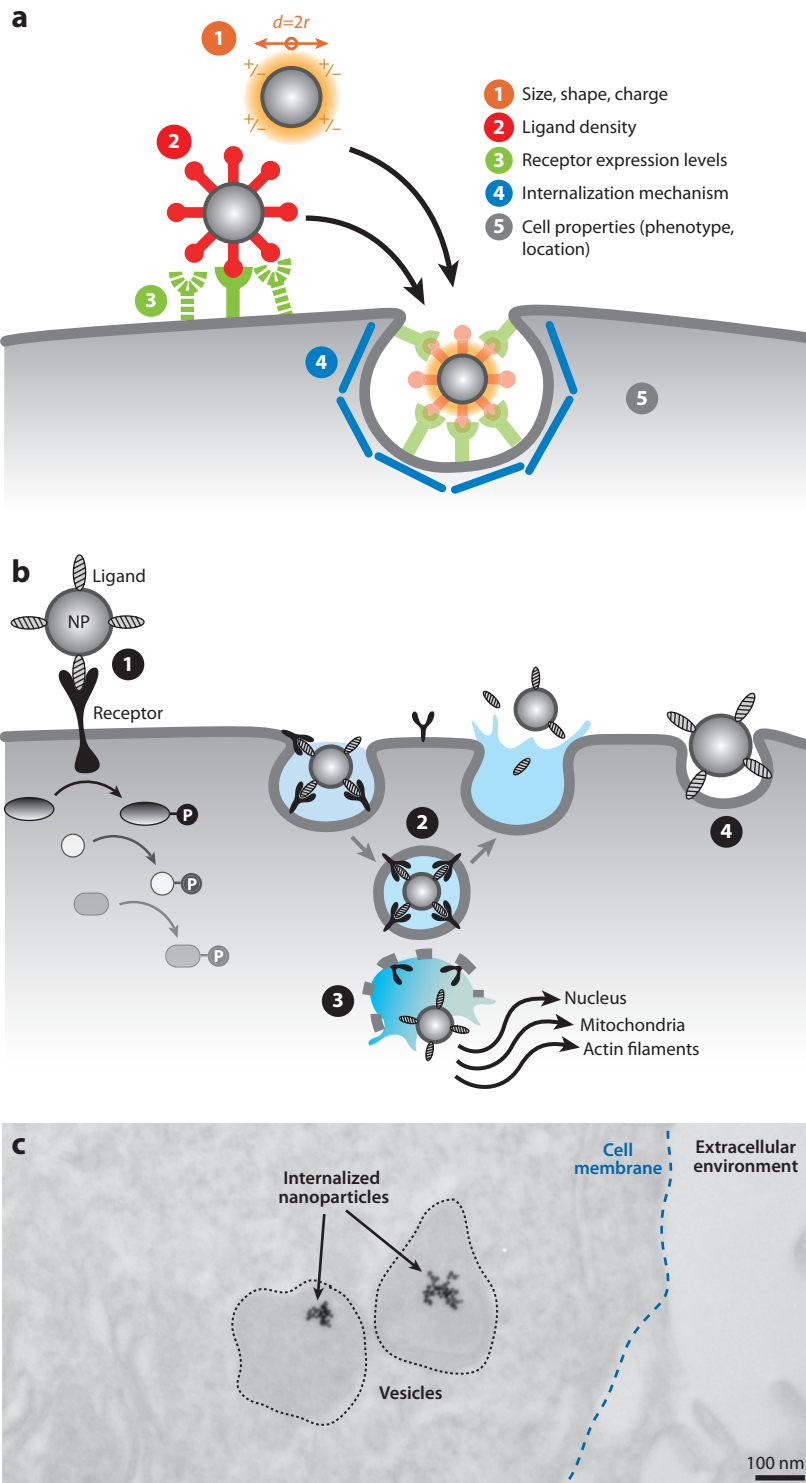
or animal models. Nanoparticles were initially considered as benign carriers, but multiple studies have demonstrated that their interaction with serum proteins and cell membrane receptors is determined by the nanoparticle design, in effect influencing cell uptake, gene expression, and toxicity. Nanoparticles can interact with the cell surface membrane in multiple scenarios, as shown in **Figure 2**.

Interactions between nanoparticle-bound ligands and cellular receptors depend on the engineered geometry and the ligand density of a nanomaterial. The nanoparticle acts as a scaffold whose design dictates the number of ligands that interact with the receptor target. A multivalent effect occurs when multiple ligands on the nanoparticle interact with multiple receptors on the cell. The binding strength of complexed ligands is more than the sum of individual affinities and is measured as the avidity for the entire complex. This phenomenon is observed in antibodies that possess at least two antigen-binding sites. Similarly, on nanoparticle surfaces, the presence of ligands at a given density over a specific curvature will contribute to the overall avidity of the nanoparticle-linked ligands for available cell receptors. To illustrate this phenomenon, the binding affinity of Herceptin to the ErbB2 receptor is 10^{-10} M in solution, 5.5×10^{-12} M on a 10-nm nanoparticle, and 1.5×10^{-13} M on a 70-nm nanoparticle (5). This example illustrates how a ligand's binding affinity increases proportionally to the size of a nanoparticle owing to a higher protein density on the nanoparticle surface. However, when viewed in terms of the downstream signaling via the ErbB2 receptor, 40–50-nm gold nanoparticles induced the strongest effect, suggesting that other factors beyond binding affinity must be considered. Nevertheless, several studies have shown that nanoparticle design can cause differential cell signaling when compared with free ligand in solution. For example, the aforementioned 40–50-nm Herceptin-coated gold nanoparticles altered cellular apoptosis by influencing the activation of caspase enzymes (5). Similarly, receptor-specific peptides improved their ability to induce angiogenesis when conjugated to a nanoparticle surface (6). Presentation of the peptide on a structured scaffold increased angiogenesis, which is dependent on receptor-mediated signaling. These findings highlight the advantages of having a ligand bound to a nanoparticle as opposed to its being free in solution. The nanoparticle surface creates a region of highly concentrated ligand, which increases avidity and, potentially, alters cell signaling.

However, this benefit comes at a cost: Nanomaterials can also cause unexpected changes in cell signaling. For example, nanoparticles coated with intercellular adhesion molecule I (ICAM-I) protein are internalized. This is an unusual finding because ICAM-I is not known to trigger endocytosis. Yet, packing multiple ICAM-I proteins onto a nanomaterial's surface produces unexpected uptake (7). Another study showed that 14-nm carbon nanoparticles interact with epidermal growth receptors and $\beta 1$ -integrins on rat alveolar II epithelial cells and induce the activation of the Akt signaling pathway, causing cell proliferation (8). An additional concern with nanoparticle-ligand complexes is the potential denaturation of proteins when bound to the engineered surface. The denaturation of a protein can affect binding to its receptor, increase nonspecific interactions or

Figure 2

Nanoparticle-cell interactions. (a) List of factors that can influence nanoparticle-cell interactions at the nano-bio interface. (b) Ligand-coated nanoparticles interacting with cells. ❶ The ligand-coated nanoparticles bind to receptors on the membrane and induce a signaling cascade without entering the cell. ❷ The ligand-coated nanoparticles can also be internalized and exocytosed by the cell, without ever leaving the vesicle. They bind to the membrane receptor, enter the cells, and then exit from the cell. ❸ Internalized nanoparticles can escape the vesicle and interact with various organelles. They bind on membrane receptors, enter the cell, and target subcellular structures. ❹ Nanoparticles can interact nonspecifically with the cell surface membrane. They are subsequently internalized. (c) Multiple transferrin-coated 15-nm gold nanoparticles are internalized by HeLa cells into intracellular vesicles. Abbreviation: NP, nanoparticle.



provoke inflammation. Lysozyme, for example, when bound onto gold nanoparticles will denature and interact with other lysozyme molecules and produce protein-nanoparticle aggregates (9). Fibrinogen also unfolds when bound to the surface of polyacrylic acid-coated gold nanoparticles. The denatured fibrinogen can then bind to the integrin receptor Mac-1 and lead to inflammation (10).

In many cases, nanoparticles enter the cell after binding to the receptor target. Once bound, several factors can dictate the behavior of nanomaterials at the nano-bio interface (**Figure 2**). For instance, a nanoparticle's shape directly influences uptake into cells: Rods show the highest uptake, followed by spheres, cylinders, and cubes (11). Gratton et al. (11) determined this ordering using synthesized nanoparticles larger than 100 nm. In studies with sub-100-nm nanoparticles, spheres show an appreciable advantage over rods (12, 13). In fact, at this size range, increasing the aspect ratio of nanorods seems to decrease total cell uptake. Although few studies have focused on nonspherical nanoparticles thus far, research indicates their interactions with cells may be much more complex. Ligand-coated rod-shaped nanoparticles may present to the cell with two different orientations. Compared with the short axis, the long axis will interact with many more cell surface receptors (14). For spiky nanostructures such as gold nanourchins, whether the ligand is located on or between the spikes affects how it is presented to the target cell receptors (15). For the engineering process, asymmetrical nanoparticles may provide another level of control in presenting ligands to the target receptors.

Within a given geometric shape, a nanomaterial's dimensions are a strong determinant of total cell uptake. For spherical gold nanoparticles, silica nanoparticles, single-walled carbon nanotubes, and quantum dots, a 50-nm diameter is optimal to maximize the rate of uptake and intracellular concentration in certain mammalian cells (14, 16, 17). In addition to size and shape, the composition of the nanomaterials also affects uptake because single-walled carbon nanotubes and gold nanoparticles, each 50 nm in diameter, possess endocytosis rates of 10^{-3} min^{-1} and 10^{-6} min^{-1} , respectively. This 1,000-fold difference may be due to the intrinsic properties of carbon versus gold. Which ligand is used to coat the nanomaterial will also affect downstream biological responses. For example, the uptake and cytotoxicity of nanoparticles were significantly altered when the nanoparticles were coated with two different proteins targeting the same receptor (18).

Once bound to their receptor, nanoparticles will typically enter the cell via receptor-mediated endocytosis (5, 13, 19). The binding of the nanoparticle-ligand conjugate to the receptor produces a localized decrease in the Gibbs free energy, which induces the membrane to wrap around the nanoparticle to form a closed-vesicle structure (19). The vesicle eventually buds off the membrane and fuses with other vesicles to form endosomes, which fuse with lysosomes where degradation occurs. The size-dependent uptake of nanoparticles is likely related to the membrane-wrapping process. Small nanoparticles have less ligand-to-receptor interaction than do larger nanoparticles. A 5-nm nanoparticle coated with 50-kDa proteins may interact with only one or two cell receptors. By contrast, a 100-nm nanoparticle has many more ligand-receptor interactions per particle. Several small ligand-coated nanoparticles must bind to receptors in close proximity to one another to produce enough free energy to drive membrane wrapping. Larger nanoparticles can act as a cross-linking agent to cluster receptors and induce uptake. Thermodynamically, a 40–50-nm nanoparticle is capable of recruiting and binding enough receptors to successfully produce membrane wrapping. Above 50 nm, nanoparticles bind such a large number of receptors that uptake is limited by the redistribution of receptors on the cell membrane via diffusion to compensate for local depletion. Nanoparticles larger than 50 nm bind with high affinity to a great number of receptors and may limit the binding of additional nanoparticles. Mathematical modeling of this phenomenon has demonstrated that optimal endocytosis occurs when there is no ligand shortage on the nanoparticle and no localized receptor shortage on the cell surface (20).

This “sweet spot” occurs in nanoparticles 30 to 50 nm in diameter where ligand density is optimal. Unfortunately, studies elucidating the effect of nanoparticle diameter on uptake were conducted primarily on immortalized cell lines. Because each cell type possesses a unique phenotype, optimal nanoparticle uptake size may depend on the cell being assayed (e.g., immortalized HeLa versus primary macrophage cells). Each cell type can express varying levels of target receptor and can utilize different internalization pathways. Thus, it will be necessary to expand nanoparticle studies to include both immortalized and primary cells in different cell culture configurations (monolayer versus three dimensional). Only then will we be able to identify broad-scope design parameters for optimal uptake and accumulation in cells.

The behavior of nanoparticles in endolysosomal vesicles remains a mystery. Some evidence suggests that the nanoparticle ligands are cleaved by the protease Cathepsin L inside endolysosomal vesicles (21). In macrophages, quantum dots seem to remain in intracellular vesicles for an extended period of time as degradative enzymes slowly decompose the core structure (22). Additional evidence suggests that compartmentalization of CdTe quantum dots into different subcellular organelles depends on size and cell type: Sub-2.1-nm quantum dots enter the nucleus, whereas 4.4-nm quantum dots are found in the cytoplasm (23). The localization of nanoparticles in the intracellular space may be directed using peptides such as the mitochondrial localization sequence. If a nanoparticle is engineered to escape the endolysosomal system, it can enter the cytosol, where it is free to interact with a wide number of organelles and can affect cell behavior. Once in the cytosol, nanoparticles can elicit biological responses by disrupting mitochondrial function, eliciting production of reactive oxygen species and activation of the oxidative stress-mediated signaling cascade (24). The production of reactive oxygen species can have detrimental effects on the mitochondrial genome, induce oxidative DNA damage, and promote micronuclei formation (25). Furthermore, certain types of nanoparticles can induce nuclear DNA damage, leading to gene mutations, cell-cycle arrest, cell death, or carcinogenesis. Demonstrating the latter, hydrophilic titanium oxide nanoparticles are oncogenic in various experimental setups (26). Once in the cytoplasm, nanoparticles will persist unless they are sorted back into the endolysosomal system where they can be exocytosed. Nanoparticles that persist in the cytosol during mitosis will be distributed within the daughter cells (27). However, the effect of the nanoparticles on subsequent cell generations remains unclear. To date, there is no consensus on the toxicity and properties of nanoparticles inside the cytoplasm. When compared with other sizes, 30-nm amorphous TiO₂ and 15-nm silver nanoparticles induce the highest generation of reactive oxygen species (28, 29). However, in other studies, the effect of nanoparticle size does not appear to be significant. For instance, the uptake of silver nanoparticles and quantum dots into macrophages induces the expression of inflammatory mediators such as TNF- α , MIP-2, and IL-1 β independent of size (22, 28). Additional work is still required before researchers fully understand the fate and toxicity of internalized nanoparticles.

In addition to a nanomaterial’s size, shape, and ligand density, surface charge is also important in dictating cellular fate. Compared with nanoparticles with a neutral or negative charge, positively charged nanoparticles are taken up at a faster rate (30, 31). It has been suggested that the cell membrane possesses a slight negative charge and cell uptake is driven by electrostatic attractions (17, 18). A recent study demonstrated that this electrostatic attraction between membrane and positively charged nanoparticles favors adhesion onto the cell’s surface, leading to uptake. For small nanoparticles (2 nm), a positive charge can perturb the cell membrane potential, causing Ca²⁺ influx into cells and the inhibition of cell proliferation (32). For larger nanoparticles (4–20 nm), surface charge induces the reconstruction of lipid bilayers (33). Binding of negatively charged nanoparticles to a lipid bilayer causes local gelation, whereas binding of positively charged nanoparticles induces fluidity. Several studies have confirmed the pivotal role surface charge plays in downstream biological responses to nanoparticles. It is important to remember that, in the

presence of serum or other biological environments, the nanomaterial's surface charge is quickly covered by a corona made up of multiple proteins. Because the surface charge affects corona composition, studies comparing positively and negatively charged nanoparticles may be describing the effects of corona composition. This phenomenon was observed when negatively charged citrate-capped gold nanoparticles were incubated with cells in culture (13, 14). Another example using negatively charged DNA-coated nanoparticles shows their interaction with serum proteins is responsible for cell uptake (34).

Nanoparticle Behavior in Live Animals

Over the past 20 years, animal-model experiments assessing pharmacokinetics and tissue distribution of various nanoparticle formulations have established some general guidelines for the effective design of nanoparticles. For instance, blood half-life is highest for neutral nanoparticles. Positively charged nanoparticles are cleared most quickly from the blood and cause several complications such as hemolysis and platelet aggregation. The unequal half-lives of different charges are a result of the interactions of nanoparticles with serum proteins such as immunoglobulin, lipoproteins, complement and coagulation factors, acute phase proteins, as well as metal-binding and sugar-binding proteins (35). These proteins instantaneously bind onto the nanoparticle surface and dictate the long-term fate, metabolism, clearance, and immune response (36). The architecture of these adsorbed proteins on the nanoparticle surface is complex but can be described as hard and soft corona layers. The hard corona layer contains proteins that are strongly adsorbed to the nanoparticle surface ($K_d \sim 10^{-6}$ to 10^{-8} M) (37), whereas the soft corona layer contains serum proteins that weakly interact with the hard corona layer. This outermost layer is likely to be dynamic and could vary during the course of the nanoparticle's life in vivo. The nanoparticle surface chemistry appears to determine the type of proteins adsorbed onto the surface and the strength of the interaction (36). Positive nanoparticles are quickly adsorbed by serum proteins onto their surface that "tag" them for removal by the mononuclear phagocyte system (MPS) inside the liver and spleen.

Most nanomaterials, when administered into the blood, are taken up within minutes or hours by the phagocytic cells of the MPS. This rapid clearance can be avoided by adding poly(ethylene) glycol (PEG) to the surface of nanomaterials. By preventing opsonization, the addition of PEG drastically increases the blood half-life of all nanomaterials regardless of surface charge. Generally, the blood half-life of gold nanoparticles is also increased by increasing the length of PEG, which causes the protective layer to thicken (38). The synthesis of these long-circulating "stealth" nanoparticles improves accumulation in the target tissue. In addition to PEGylation of nanoparticle surfaces, blood half-life also depends on a nanoparticle's shape, size, and surface chemistry. For example, rod-shaped micelles have a circulation lifetime ten times longer than that of spherical micelles (39). For intravenously administered nanoparticles, diameter is an important determinant of pharmacokinetics and biodistribution owing to the variable size of interendothelial pores lining the blood vessels. Nanoparticles with diameters smaller than 6 nm are quickly eliminated from the body because they can be excreted by the kidneys (40). Unless a nanomaterial consists of degradable materials such as polymers, lipids, or hydrogels, it cannot be eliminated by the kidneys when the diameter is greater than 6 nm. Nanoparticles with diameters larger than 200 nm accumulate in the spleen and liver, where they are processed by the MPS cells.

Nanoparticles can also accumulate in tumors and be used to deliver therapeutic compounds or contrast agents for imaging (**Figure 3**). Tumors possess large fenestrations between the endothelial cells of blood vessels produced by angiogenesis and can retain particulates found in the blood. This response, termed enhanced permeation and retention (EPR), allows nanoparticles to

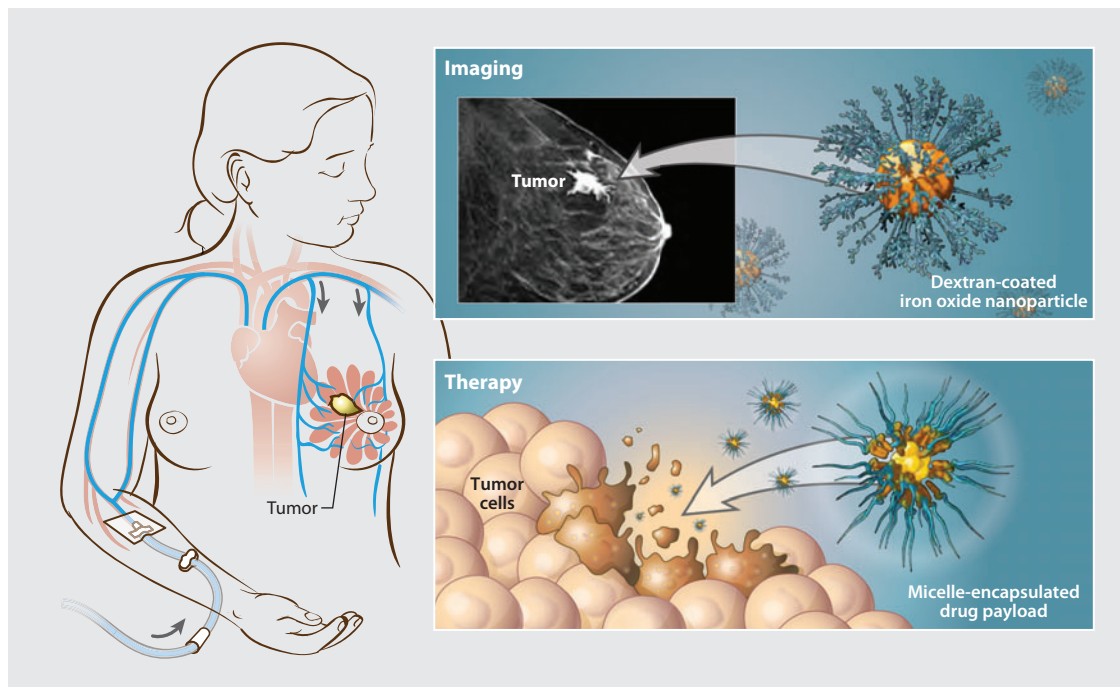


Figure 3

Nanoparticles in tumor-specific delivery. Nanoparticles can be injected into a patient's blood and accumulate at the site of the tumor owing to enhanced permeation and retention. This preferential accumulation at the tumor can be used to deliver contrast agents such as dextran-coated iron-oxide nanoparticles for magnetic resonance imaging or therapeutic compounds such as micelles carrying chemotherapeutic drugs. We acknowledge AXS Biomedical Animation Studio (Toronto, Ontario, Canada) for drawing this schematic.

accumulate inside the tumor if they are not cleared by the liver or spleen or excreted through the kidney. To produce long-circulating nanoparticles that can accumulate inside tumor tissues a diameter between 30 nm and 200 nm is desired (41). Using this approach, researchers may induce passive accumulation of nanomaterials inside a tumor. In fact, it is possible to control the overall accumulation and penetration depth into the tumor by changing a nanoparticle's diameter (38). Researchers determined that the nanoparticle's capacity to navigate between the tumor interstitium after extravasation increased with decreasing size. By contrast, larger nanoparticles (100 nm) do not extravasate far beyond the blood vessel because they remain trapped in the extracellular matrix between cells. Thus, the smallest nanoparticles (20 nm) penetrate deep into the tumor tissue but are not retained beyond 24 h. Surprisingly, adding a targeting moiety on the surface of the nanoparticles does not appear to increase accumulation inside the tumor or change biodistribution. Active targeting of nanoparticles changes the intratissue localization of nanoparticles and their increased internalization into cancerous cells (42, 43). However, some studies have shown active targeting increases tumor accumulation of 20-nm nanoparticles. Owing to these contradictory findings, it is currently uncertain how active targeting affects nanoparticle accumulation in the target tissue (43).

Unfortunately, tumor accumulation always represents a mere fraction of total injected dose (1–10%): The majority of injected nanomaterials end up in the liver and spleen. Resident MPS macrophages will phagocytose nanoparticles, degrade a small fraction of them, and eventually

exocytose both the degraded and intact nanoparticles (22). The toxicity, properties, and fate of these exocytosed nanoparticles have not been determined. If a nondegradable nanomaterial is too large to be excreted via the kidneys, it will remain in tissues inside the body for up to 8 months (44). Researchers have not evaluated the longer-term distribution (> 1 year) of nanoparticles, which raises concerns about toxicity. CdSe quantum dots exhibit hepatotoxicity in cell cultures but do not appear to be toxic in Sprague-Dawley rats on the basis of histopathology as well as liver and kidney marker analysis (45). In addition, pristine and PEGylated single-wall carbon nanotubes were nontoxic to rats and rabbits despite their reported toxicity within *in vitro* culture models. The differential toxicity may be related to a localized concentration of nanoparticles (46). *In vivo*, nanoparticles seem to be continuously transported from organ to organ, whereas, *in vitro*, the nanoparticles are located within a limited space. Therefore, the differential toxicity between *in vivo* and *in vitro* may be due to exposure concentration.

Many questions remain unanswered regarding the *in vivo* behavior of nanoparticles. For example, how does protein opsonization impact the kinetics of nanoparticles? How are nanoparticles metabolized? What is the long-term fate of nanoparticles? How do the physicochemical properties of nanomaterials affect their biodistribution behavior *in vivo*? Addressing these questions will be important because the only way to improve the design of biomaterials is to fully elucidate their effects, transformation, and final fate within biological systems.

PERSPECTIVE

Nanoparticle Design

Outcomes from studies on nano-bio interactions in the past decade have greatly influenced nanoparticle design. The evolution of nanoparticles destined for biomedical applications has occurred in parallel with studies investigating the biological responses to the nanomaterials themselves. Material design evolved whenever the effect of size, shape, or surface charge was further elucidated. To date, three generations of nanoparticles have been engineered for biomedical applications (see **Figure 4**). The first generation consisted of novel nanomaterials functionalized with basic surface chemistries to assess biocompatibility and toxicity. The second generation produced nanomaterials with optimized surface chemistries that improved stability and targeting in biological systems. The third generation shifted the paradigm of design from stable nanomaterials to “intelligent” environment-responsive systems that should improve targeted compound delivery.

The first generation of nanomaterials was synthesized to demonstrate the potential applications of novel materials in biomedical research. Before this generation, liposome studies in the suprananoscale range (100–1,000 nm) established a basic research template for evaluation of more modern, smaller materials (1–100 nm). Seminal papers from this first generation include those describing the surface modification of organic-soluble quantum dots and magnetic nanoparticles to render them water soluble and stable enough for biological applications (47, 48). First-generation nanoparticles were modified with nonstealth surface chemistries and used in experiments to assess cell uptake and toxicity (13, 49). The main focus of these materials was to determine the effects of surface charge (50, 51). However, most of these studies included experiments in serum-free media or did not account for serum-protein interactions with their nanomaterials, which makes the findings difficult to interpret when considering physiological conditions within the body. First-generation nanomaterials also did not use PEG; thus, most *in vivo* data show the rapid clearance of nanomaterials (52, 53). These early studies were important in highlighting the biocompatibility of these novel materials and marked a major step in the transition from chemical synthesis to


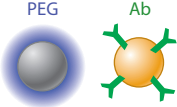
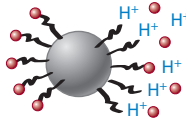
	1 st generation	2 nd generation	3 rd generation
Nano-materials	 <ul style="list-style-type: none"> • Material design • Water solubility • Biocompatibility 	 <ul style="list-style-type: none"> • Maximize delivery • Stealth (passive) • Active targeting 	 <ul style="list-style-type: none"> • Environment-responsive • Dynamic properties • Biological or external cues • Theranostic abilities
Biological challenges	<ul style="list-style-type: none"> • Unstable • Removal by MPS • Poor tumor targeting 	<ul style="list-style-type: none"> • Overreliance on EPR effect • No “universal” antigen • Active targeting is disappointing • <10% dose in tumor 	<ul style="list-style-type: none"> • To be determined

Figure 4

Evolution of nanoparticle design, highlighting the interplay between evolution of nanomaterial design and fundamental nano-bio studies. Abbreviations: Ab, antibody; EPR, enhanced permeation and retention; MPS, mononuclear phagocyte system; PEG, poly(ethylene) glycol.

biological uses. However, the poor stability in cell-culture media and rapid clearance from the body shifted research focus to increase stability and prolong blood half-life.

With nanoparticle synthesis established, research shifted to surface chemistry optimization for diagnosing and treating cancer. Although nanoparticles were a promising avenue for targeted delivery in many organs and tissues, most studies using second-generation nanoparticles focused on tumor delivery as a proof of concept. These studies relied on the EPR effect to ensure passive accumulation. Consequently, second-generation nanomaterials are characterized by two important design concepts: stealth and active targeting. The goal of stealth nanoparticles is to maximize blood circulation half-life to ensure continuous delivery of nanoparticles into the tumor via leaky vasculature. The longer a nanoparticle remains in circulation, the higher likelihood it has of entering the tumor. Several studies showed the increased half-life of nanomaterials by simply adding PEG to their surface (54). The chemistry used to attach PEG, overall PEG length, and surface density all affect nanoparticle stability (38, 55, 56). Alternative molecules such as lipids and silica were investigated, but PEG remains the most widely used approach (57). Active-targeting nanoparticles also rely on the EPR effect to access the intratumoral space. However, targeting moieties are used to potentially increase total accumulation by anchoring the nanoparticles onto the cells. Unfortunately, addition of excess targeting ligands also increases clearance by the MPS because more proteins are now “visible” on the surface than with PEG (58). However, active targeting did not produce drastic increases in tumor targeting (42, 43). Some studies show a modest increase in tumor accumulation, but the total amount of nanoparticles is always overshadowed by the large fractions retained by the liver.

Several concerns arise regarding second-generation nanomaterials. First, there appears to be an overreliance on the EPR effect to deliver nanoparticles into the tumor, although this phenomenon may not be a universal property of all tumors in human patients. Second, no single nanoparticle size can access all areas of the tumor and accumulate in significant quantities. Large nanoparticles do not extravasate far beyond the blood vessel, and small nanoparticles travel deep into the tumor but remain there only transiently. Third, the advantages of active targeting are offset by the barrier effect whereby most nanoparticles do not travel beyond the first few layers of cells because they adhere to their targeted receptors.

Responding to these concerns, researchers recently developed a new generation of nanomaterials that do not rely on passive retention of nanoparticles or on endogenous tumor ligands. The third generation of nanomaterials has “environment-responsive” properties. These dynamic nanoparticles use biological, physical, or chemical cues in their target environment to trigger a change in their properties to maximize tumor delivery. Two approaches have been used so far: The first uses hallmark cues inside the tumor environment such as low pH, low O₂, or matrix metalloproteinase enzymatic activity; the second provides an artificial cue such as near-infrared light inside the target tissue. Some examples of physiological cues include a pH-triggered deshielding of the PEG surface layer to reveal a positively charged surface that causes nonspecific uptake of drug-filled nanoparticles (59). Other approaches have used pH to trigger the detachment of drugs from a nanoparticle’s surface or to break down the nanoparticle to release drugs inside the local tumor environment (60). Enzymatic activity has also been used as a trigger for drug release or fluorescence (61, 62). One study used local tumor enzymes to degrade large gelatin nanoparticles that contained small fluorescent quantum dots. This method maximizes tumor retention while ensuring quantum dot penetration deep into the tumor for improved diagnostic sensitivity (63). Alternatively, artificial environmental cues such as near-infrared light can be used to excite nanorods or nanoshells inside the tumor to generate heat that can trigger localized drug release from the liposomes (64, 65). Recently, the concept of communicating nanoparticles was demonstrated: An initial population of “broadcasting nanoparticles” inside the tumor is used to trigger the coagulation cascade using infrared light. The “receiver” nanoparticles are designed to target specific molecules produced by the coagulation process at a high concentration (66). All these approaches elegantly circumvent the need for universal tumor antigens and do not overly rely on the EPR effect. Also, by using local cues inside the tumor to trigger drug release, the nanoparticles inside the liver and spleen do not cause toxicity.

Fundamental Interactions

The recent burst of innovative design strategies for targeted delivery of nanomaterials has been met with excitement by academia, industry, governments, and the general public (67). However, the rush to publish novel proof-of-concept studies has occurred at the expense of fundamental nano-bio studies. This lack of fundamental knowledge is catching up to the field. We are currently unable to draw specific or general conclusions regarding the impact of size, shape, and surface chemistry-dependent interactions. This subdiscipline of nanotechnology research is still young, and researchers have been evaluating different approaches for relevant studies. Many initial studies did not fully characterize the nanoparticles used, making it difficult to ascribe the biological responses observed to a specific nanoparticle design parameter. Furthermore, the interference of nanoparticles in biological assays was not considered in early studies, which impacts the current interpretation of these results. Instrumentation and technology have also progressed and allow for an increasingly thorough characterization of nanoparticles before administering them to cells and tissues. Finally, many of the first-generation nanoparticle designs were inappropriate for biological studies. For example, direct adsorption of a radioactive agent on the surface of nanoparticles to measure biodistribution may not be appropriate because radioactive agents could desorb and could have a different *in vivo* trajectory than that of the nanoparticles. Researchers are now well aware of this caveat, and they have developed new strategies to account for these experimental challenges.

Nano-bio interactions must be heavily investigated in this century so that investigators can design the most efficient nanoparticle-based delivery systems. Not only will the results influence the engineering of nanosystems, but they will also have an impact on our understanding of how biology works because most biological components are nanometer in scale. Engineered nanoparticles

offer an unprecedented opportunity to investigate the morphology and chemistry of nanoscale objects in mediating biological responses. Unlike pathogens and proteins, nanomaterials can be engineered in a reaction chamber and their physical and chemical properties can be precisely tuned to enable systematic studies. By conducting these studies in a systematic manner and with a properly selected biological system, researchers could create a database that would enable one to find commonalities in the experimental results. This could lead to the development of predictive and simulation tools to assist in the engineering process. Already, Monte Carlo simulations have been used to model the effect of nanoparticle size and ligand density on cellular uptake and tumor targeting to improve the nanoparticle design for optimal tumor accumulation for diagnostic and drug-delivery applications (68–70). Ideally, the outcomes of these studies would also be entered into a database, which would further enable practitioners to use computer-simulation programs to identify quickly the most appropriate nanostructure design for a specific application. All fundamental studies on nano-bio interactions at the systemic, cellular, and molecular levels could populate this database, making it useful in generating correlations between the physicochemical properties of nanostructures and biological responses. Ultimately, this database could create blueprints for constructing nanosystems.

CONCLUSION

The ability to engineer structures in the nanometer-size range has already demonstrated great value to the fields of optics and electronics. Before nanotechnology can significantly impact medicine, it will be important to characterize the effect of nanomaterials on biological systems. In the next decade, it will be important to elucidate how the physicochemical properties of nanomaterials and their by-products interact with subcellular organelles, cells, tissues, and organisms. This will greatly affect our ability to engineer new generations of nontoxic products containing nanoparticles. For this endeavor to be successful, focus must shift from proof-of-concept studies to thorough fundamental studies. The publication of well-executed fundamental studies will provide the design criteria for successful nanoparticle-based strategies in vivo. The progress in nanobio, material synthesis and computer simulation studies can potentially change how we engineer nanostructures in this century and could lead to novel applications.

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