International Journal of Nanomedicine

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REVIEW

Parameters and characteristics governing cellular internalization and trans-barrier trafficking of nanostructures

This article was published in the following Dove Press journal:
International Journal of Nanomedicine
18 March 2015
Number of times this article has been viewed

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Abstract: Cellular internalization and trans-barrier transport of nanoparticles can be manipulated on the basis of the physicochemical and mechanical characteristics of nanoparticles. Research has shown that these factors significantly influence the uptake of nanoparticles. Dictating these characteristics allows for the control of the rate and extent of cellular uptake, as well as delivering the drug-loaded nanosystem intra-cellularly, which is imperative for drugs that require a specific cellular level to exert their effects. Additionally, physicochemical characteristics of the nanoparticles should be optimal for the nanosystem to bypass the natural restricting phenomena of the body and act therapeutically at the targeted site. The factors at the focal point of emerging smart nanomedicines include nanoparticle size, surface charge, shape, hydrophobicity, surface chemistry, and even protein and ligand conjugates. Hence, this review discusses the mechanism of internalization of nanoparticles and ideal nanoparticle characteristics that allow them to evade the biological barriers in order to achieve optimal cellular uptake in different organ systems. Identifying these parameters assists with the progression of nanomedicine as an outstanding vector of pharmaceuticals.

Keywords: nanoparticles, transport mechanisms, cellular uptake, size, shape, charge

Introduction

The emergence of nanomedicine provides a strategic, therapeutic tool that aims to increase drug targeting to site-specific areas within the body. Nanoparticle (NP) research has identified the crossing of mucosal barriers and cellular uptake to support NP utilization, as well as NP surface properties that affect these phenomena.[1] In the design of NPs for biological use, significant factors to overcome limitations associated with insufficient drug delivery to targeted sites include NP size, surface charge, shape, chemical composition, and stability.[2,3] Manipulating these pertinent NP characteristics may facilitate various applications and enhanced cellular and trans-barrier internalization of NPs into the target sites. These sites innately have a biological barrier to prevent the entry of foreign objects, thus resulting in decreased drug concentrations at the intended site. Ideally, nanomedicine should circumvent the biological barriers and enhance drug targeting and NP uptake.[4]

Figure 1 illustrates different transport mechanisms across and into the biological membrane for the internalization of NPs; key terms related to NP internalization and trans-barriers are provided in Table 1. According to Kumari et al[5] NP internalization occurs mainly through intracellular, paracellular, and transcellular pathways. However, endocytosis pathways are poorly understood regardless of their clinical significance and continued research.[3] Continued research in this paradigm, coupled

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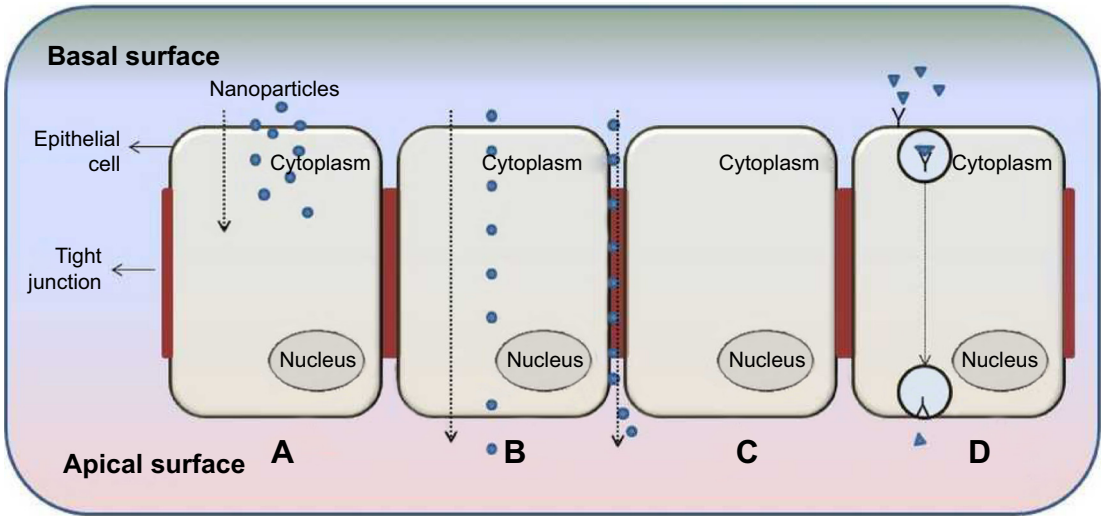

Figure 1 The transport mechanisms of a typical biological barrier.
Notes: (**A**) Cellular internalization of nanoparticle into cell via endocytosis; (**B**) transcellular transport of nanoparticles through cell; (**C**) paracellular transport of nanoparticle between cells through the tight junction; and (**D**) receptor-mediated transcytosis.

with nanoparticulate internalization and characterization, will provide immense insight into an ideal pharmaceutical formulation design.

Current studies on nanomedicine are encouraged in order to structure a framework that enables efficient, safer drug delivery and to eliminate many of the disadvantages posed by conventionally delivered drugs. Studies to specifically determine the effect of NP internalization are limited yet necessary in order to enhance biomedical technology and inform toxicity studies. Elucidating the parameters of NPs that enable them to target cells in response to disease-specific signals could significantly improve the therapeutic care of complex diseases. The current review therefore discusses NP properties and characteristics such as size, shape, charge, hydrophobicity, and ligand attachments that influence their uptake into target cells and through biological barriers. Intracellular pathways and current mechanisms employed to augment NP uptake and biological barrier transport were also discussed in detail.

Transport mechanisms of nanocarriers

Intracellular endocytic delivery pathways

Various receptor-mediated pathways exist for cellular internalization of biological substances such as hormones and enzymes that require internalization to exert an effect at a cellular level (Figure 2). By adopting these mechanisms, drugs and NPs can be delivered to the necessary cell type. Cellular uptake mechanisms need to be understood in order to enhance internalization and identify NP characteristics that promote specific mechanisms.[1] The mechanisms of different endocytic pathways as illustrated in Figure 1A are thoroughly described in the subsequent discussions.

Pinocytosis

Included in the pinocytosis classification are clathrin- and caveolae-mediated endocytosis and macropinocytosis. Clathrin-mediated endocytosis involves clathrin-coated vesicle formation in the presence of adaptor and accessory proteins.

Table 1 Key terms

Term	Definition
Cellular internalization	Process by which biological and foreign matter is taken up by cells.
Endocytosis	Energy or enzyme-dependent mechanism of cellular internalization.
Trans-barrier	Refers to transport of nano- and micro-substances through cells from extracellular fluid through the apical and basolateral membrane.
Opsonization	Biological phenomenon whereby opsonin molecules adsorb onto the surface of foreign particles to enhance RES recognition and phagocytosis.
PRINT particles	Particles fabricated using a lithographic technique of PRINT to produce monodisperse, shape-controlled particles.

Abbreviations: PRINT, particle replication in non-wetting templates; RES, reticuloendothelial system.

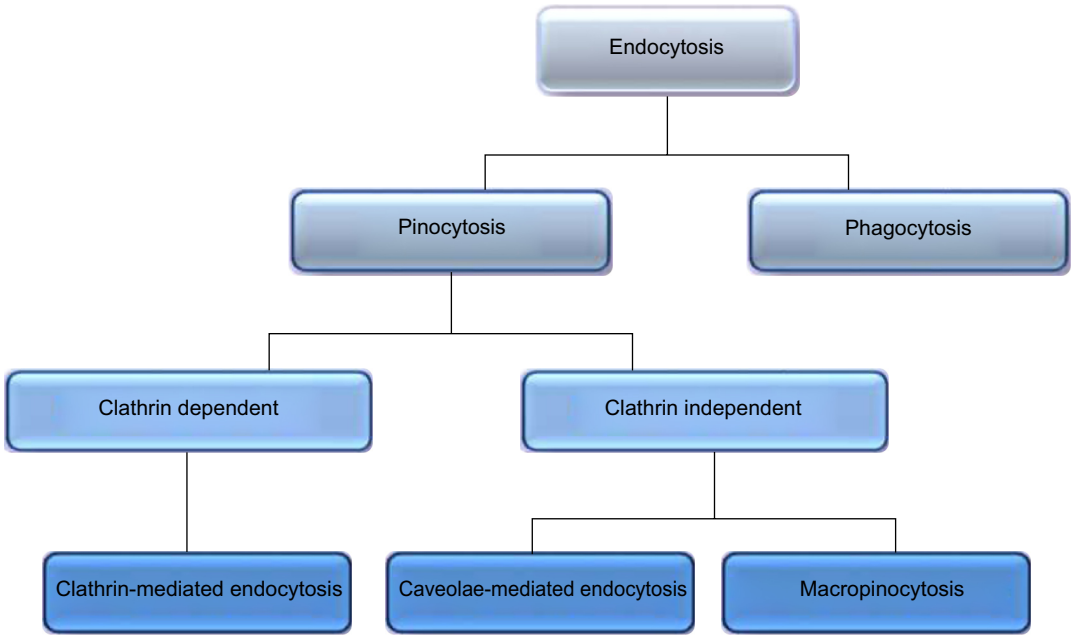

Figure 2 Mechanisms of endocytosis subdivided into categories of cell uptake.

Endocytic event cascade is activated by the signaling of the NP on the cell surface,[6] which aligns surface proteins to prompt clathrin recruitment from the cytosol to begin clathrin-coating on the inner membrane of the cell. An adaptor protein, Epsin, is involved in the initial stages of membrane curvature and pit formation and accessory proteins such as dynamin (GTPase) affect vesicle formation from shallow to deep invagination by inducing deformation of the membrane.[7] With the aid of dynamin, a clathrin-coated vesicle with a size of 100–150 nm is formed due to polymerization of the coat complex and the NP-containing clathrin-coated vesicle then internally detaches from the donor membrane.[6] Once within the cell, clathrin and adaptor proteins uncoat to allow fusing of the vesicle within the cell to release the endocytosed NPs (Figure 3).

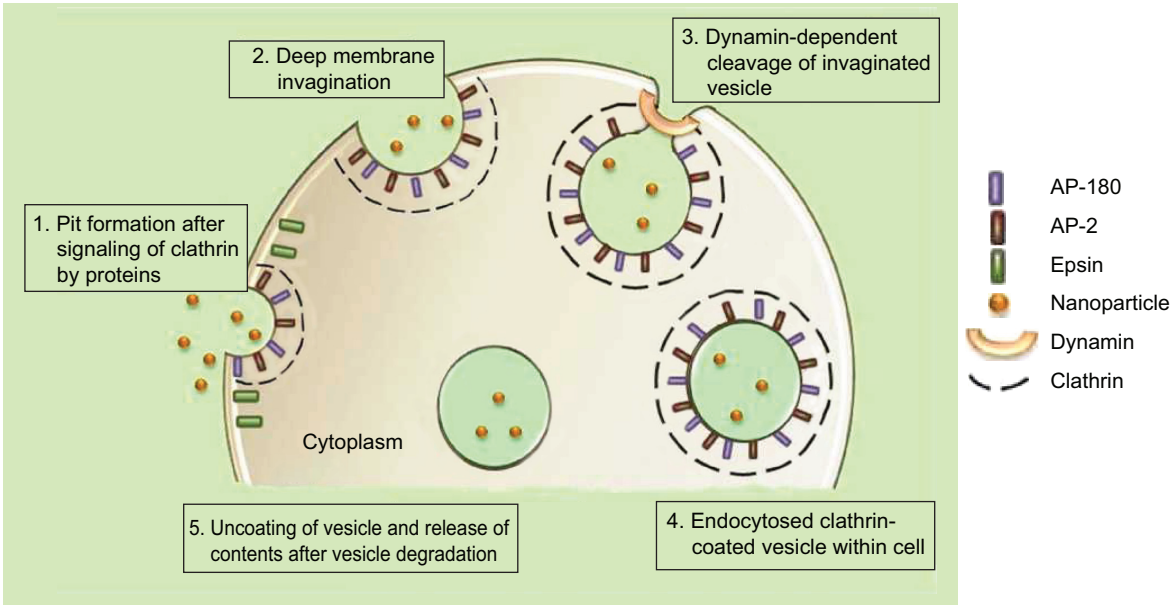

Figure 3 Mechanism of clathrin-mediated endocytosis of nanoparticles.

Caveolae-mediated endocytosis is a pathway dependent on membrane cholesterol, dynamin, and cell receptor mediation.[8] Caveolae are formed by a cluster of caveolin proteins (caveolin-1, -2, -3) that bind directly to membrane cholesterol,[9] as shown in Figure 4. Complex signaling is driven by the cell membrane–bound NPs to be endocytosed, which induces actin reorganization and dynamin recruitment from the cytosol[10] to stimulate membrane invagination and further on, vesicle budding. The uncoated invagination initially assumes a flask shape with a body diameter of 60–80 nm and a neck diameter of 10–15 nm.[11] Similar to clathrin-mediated endocytosis, dynamin regulates vesicle budding of the caveolae to internalize cell membrane segments that contain the cargo and the caveolae membrane fuses into the acceptor compartment to release its contents.

Macropinocytosis

Macropinocytosis is a clathrin-, caveolin-, and dynamin-independent process that involves uptake of a larger volume of the membrane, also allowing for larger sized particles of sizes >1 μm to be internalized.[12] The pathway proceeds by forming protrusions due to actin polymerization from the cell membrane, which then encapsulates the substance to be internalized and once again fuses back with the cell membrane.[9] The process is initiated by activation of tyrosine kinases that signal a cascade of changes in the actin cytoskeleton and the formation of membrane protrusions. The random macropinocytic extensions enclose the material in the extracellular environment for cellular uptake (Figure 4) and collapse to fuse with the cell membrane, generating an internalized macrosome vesicle of an average of ~10 μm.[12,13] The uncoated membrane of the macrosome either acidifies and shrinks or fuses with the lysosomal compartment, consequently releasing the NPs into the intracellular compartment.[14]

Phagocytosis

Similar to macropinocytosis, phagocytosis is also a clathrin-, caveolin-, and dynamin-independent process and is proficient in internalizing larger particles such as drug nanocarriers and pathogens.[14] It involves sequential instigation of receptors as a result of cell surface recognition of cargo, leading to internalizations by encircling it into triggered cup-shaped cell membrane deformations, forming a phagosome.[9] The pathway begins with opsonization of the NPs, which then attach to the cell surface via Fc receptors and complement receptors,[15] marking the beginning of actin polymerization and rearrangement and membrane extension to form surface extensions that encapsulate the opsonized NPs for internalization (Figure 4).[14,16] A reduction and contraction of actin at the base of the cup allows for closure of the phagosome and the entire actin-lined phagosome is then internalized with its contents.[15] In a macropinocytotic manner, the vesicle undergoes degradation after transporting the opsonized particle into the cell.

Transcellular delivery pathway

As described by clathrin-dependent and clathrin-independent internalization, postuptake, the vesicle undergoes degradation. However, through other pathways, the vesicle can be transported to the other end of the cell surface, where its contents can be expelled into the extracellular environment as shown in Figure 1B.[17] This pathway allows for lipophilic NPs and molecules to move efficiently through the transcellular route

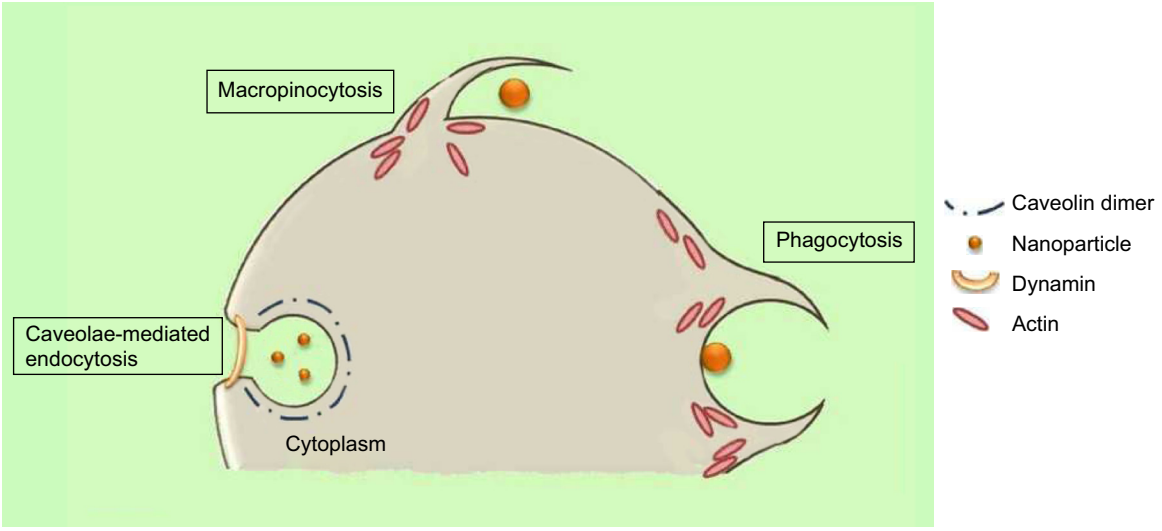

Figure 4 The mechanisms of caveolin-mediated endocytosis, macropinocytosis, and phagocytosis.

by partitioning into and out of lipid bilayers via vesicular carriers due to its lipidic nature.[18] The cargo to be transported binds to cell membrane receptors to form a complex. Membrane invagination is then initiated and the deep pit is internalized as a vesicle containing NPs.[19,20] Following internalization, the endocytosed vesicle is sorted into a transcytotic vesicle to prevent typical endosome degradation. The transcytotic vesicle is then transported to the other end of the cell, where the vesicle membrane fuses with the cell membrane and the content of the vesicle is secreted externally.[19,21]

Paracellular delivery pathway

Paracellular delivery of drugs is a passive process that occurs between adjacent cells via tight junctions (TJs) through the intercellular space and not through the cell as described by transcellular delivery (Figure 1C).[22] This route is specific for hydrophilic NPs and molecules, since hydrophilic molecules cannot cross biological membranes and as aqueous pathways that normally absorb nutrients, vitamins, or cofactors.[18] Hydrophilic molecules are unable to cross biological membranes in the manner of lipophilic molecules due to the lipid properties of the cell bilayer membrane; therefore, targeting the paracellular pathway can significantly affect their transepithelial transport. Transit through this route is regulated by TJs that have heterogeneous pores with a variety of diameters of up to 15 Å;[22] the overall charge of the TJ is negative and is therefore more responsive to positively charged NPs for paracellular permeation.[23]

Parameters and characteristics of nanostructures governing cellular internalization

The need for understanding the fundamental physicochemical characteristics of NPs and their pivotal role in cellular uptake is essential when designing smart nanosystems (Graphical Abstract). Ascertaining NP functionality provides basic and necessary information to influence the rational design of optimal nanocarriers by selectively delivering drug to targeted tissue to increase the rate of cellular uptake and effectively reduce drug-induced side effects.[24–26] Other determining factors relating to cellular uptake are: 1) variation in the uptake pathways,[27] 2) cell specificity,[28] 3) NP interaction with cell surface receptors,[25] and 4) NP interaction with plasma proteins leading to NP–protein complex formation.[29]

Particle size

An important consideration regarding particle size is the 4 μm diameter of the smallest blood vessels, as a micrometer-sized particle may cause an embolism. Acknowledging size should therefore not be limited to its internalization ability but also for its physical effects once inside the body.[30,31] Cells within the human body vary from 1–100 μm, and therefore, have a low probability of internalizing particles of ~100 μm. This particle size range for intracellular delivery is limited to the cells with a large enough capacity to host the particle. In addition to the higher rates of endocytosis of smaller sized NPs (<100 nm),[12,32,33] experimental data also promote higher bioavailability of the endocytosed drug carrier at 100–1,000 nm.[34,35] Various studies revealed that within a 1–100 nm range, 50 nm NPs show maximum cellular uptake, with 14–20 nm NPs having a higher endocytotic rate than the 100 nm NPs.[36–38] No significant difference in cellular uptake has been shown by NPs between 25–130 nm,[39] while some reports postulate that NPs have higher internalization between 50–100 nm.[40] On the contrary, from a cellular uptake research experiment using thio-organosilica NPs of 50–500 nm, Awaad et al[38] concluded that 95–200 nm is the ideal size for increased cellular uptake.

In an in vitro study undertaken by Nicolete et al[41] after 4 hours of being culture incubated, 6.5±3.9 μm poly(lactic-*co*-glycolic acid) (PLGA) microparticles were still attached to the cell surface and required more time for endocytosis to occur. Simultaneously, PLGA NPs of size 389 nm (polydispersity index =0.2) had already, within the same time period, been encapsulated in vesicles and endocytosed into the intracellular compartment (Figure 5). These results were consistent with the study conducted by Loh et al[42] which demonstrated poor internalization capability of chitosan particles that were >1 μm, much unlike the extensive uptake of NPs of sizes 110–390 nm. Interestingly, Sahay et al[12] stated that microparticles also enter cells but not as rapidly as NPs and concluded that poly(ethylene glycol) (PEG) particles <5 μm can gain entry into cells via pinocytosis. Likewise, considering the possibility of the aggregation of NPs, the size of an aggregated nanocluster is larger than a single NP and will affect the internalization rate accordingly. In vitro, the stability of the NPs should be controlled to promote a consistent nanosystem.

A research review compiled by Acosta[34] predominantly concluded that NPs with a size of ≤500 nm produce higher cellular uptake than NPs of a larger size. These results were also coherent with the enhanced penetration abilities of specialized nanocarrier systems that housed the NPs. The uptake of NPs with a size of 500 nm has a lower viability but may be promoted with the use of a constructive, complementary delivery system. Essentially, an assisting

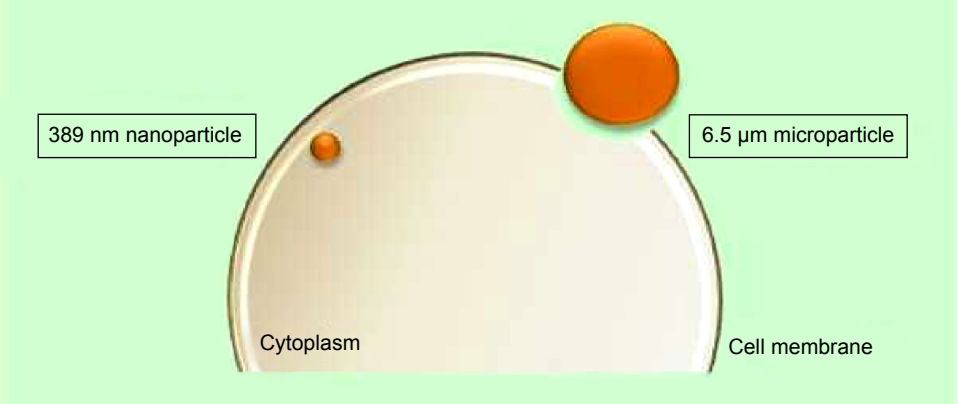

Figure 5 Representation of the internalization potential dependent on particle size.
Note: The larger surface area of the nanoparticle allows for increased surface contact with the cell membrane for higher internalization rates as described in the investigation of Nicolete et al.[41]

delivery system would have lipophilic properties or a suitable surface charge for enhanced internalization.

Despite several studies promoting the use of smaller NPs (<100 nm), disparities exist in other studies providing evidence that particle size affects internalization as significantly as its complementary system. The variation in internalization profiles and NP size demonstrates that the influence of NP size is also dependent on the type of cells and the chemical composition of the nanomaterial.[12,42]

Surface charge

Charge parameters are vital in the characterization of NPs, as they determine aggregation in the blood or interaction with oppositely- and like-charged cell membrane surfaces. A plethora of studies concluded that cationic and neutral particles show the highest transport efficiency compared to negatively charged particles due to the charge attraction between the positive NPs and negative cell membrane surface, thereby increasing the rate and extent of internalization.[33,43–45] These electrostatic forces are long-range forces and can act across intervening aqueous space.[46]

Cationic and neutral nanoparticles

In the study by Jallouli et al[47] the uptake of 60 nm neutral and cationic maltodextrin porous NPs with a phospholipid core was investigated in brain capillary endothelial cells. Neutral NPs were endocytosed in the caveolae-mediated pathway, while the cationic NPs were found to utilize the paracellular pathway due to the rich anionic sites found on the luminal surface of the endothelial cells. The cationic NPs had a stronger charge affinity to the collagen fibers on the surface of the endothelial cells, thus impeding drug delivery, while the neutral NP sample showed better transcytosis into the targeted cells.

Cationic nanoparticles

Karlsson et al[48] showed that cationic particles had a 2.5-fold higher uptake than neutral particles and a 25-fold higher uptake than anionic particles, concluding that cationic particles are transported using the paracellular pathway to a greater degree than neutral or anionic particles. These results contradict the study by Lin et al[49] who concluded from their experimental study that cationic and neutral gold-coated NPs were internalized via endocytosis while anionic NPs showed lesser permeability and were trafficked through the paracellular pathway.

Anionic nanoparticles

Contrary to the abovementioned research, many studies have shown the successful internalization of negatively charged NPs. An interesting study conducted by Harush-Frenkel et al[43] concluded that both anionic and cationic PEG-D-L-polylactide (PLA) NPs accumulated within MDCK and HeLa cells. The internalized anionic NPs underwent a degradative lysosomal process and were unable to undergo further transcytosis, rendering cationic NPs more suitable for cellular drug delivery. Likewise, negatively charged quantum dot NPs were, in fact, endocytosed through a caveolae-mediated pathway, while cationic quantum dot NPs used a clathrin-mediated pathway, internalizing HEK cells.[50] Both charged quantum dots were internalized via endocytosis with no specific rationalization to endocytic preference. Despite the hypothesis of NPs with a negative charge having a slower uptake rate due to repulsive forces,

research has shown that certain anionic NPs internalize more readily. From this discussion, it can be deduced that anionic NPs have the ability to undergo internalization via caveolae pathways, whereas cationic NPs commonly use the clathrin pathways.[43,50] Highly charged negative NPs also favor good stability since the Coulombic repulsion forces arising from their surface charge can overcome the Van der Waals attractive forces between them and prevent aggregation.[51]

Particle shape

Functional behavior and internalization of particles in drug delivery are strongly influenced by their shape.[52] Although few researchers have focused on shape, those who have, have made noteworthy contributions, but with contradicting results.[53] In many of the following studies, NP shape was the key factor for enhanced internalization, proving its pivotal role in NP fabrication. Chithrani et al[36] investigated the uptake of gold spherical and rod-shaped NPs. The 74 nm and 14 nm spherical NPs had a higher uptake when compared to 74×14 nm rod-shaped NPs by 500% and 375%, respectively, which support their claim that spherical particles have a higher internalization probability. The speculation is that the difference in curvature between the two shapes determines cell surface binding. When the longitudinal axis of the rod-shaped NP is in contact with the cell surface, it has a larger area of contact with the cell membrane receptors compared to spherical NPs, and therefore blocks the remaining available membrane receptors, reducing the number of NPs being internalized. Han et al[54] have reported similar results that prove that spherical particles internalize substantially quicker than asymmetrically shaped particles. Champion and Mitragotri[55] reported on the correlation between contact angle and particle internalization, concluding that rod-shaped NPs have a higher likelihood of internalization when their major axis is perpendicular to the cell membrane. The long axis of the rod aligned perpendicular to the cell will increase the internalization rate and the rate will decrease as ø increases (Figure 6). This theory is based on the orientation of the NP to the cell membrane and could further dictate the synthesis of NP shapes with several short aspects to enhance internalization.

Another theory was reported by Gratton et al[57] through a study using various shaped PEG-based PRINT (particle replication in non-wetting templates) particles. Among different shapes investigated, nano-cylinders were internalized to a considerably larger extent than micro-cylinders and nano-cubes. The higher cell uptake was speculated to be due to the larger surface area, allowing for more multivalent ionic interactions with the cell membrane, which then undergo endocytosis and phagocytosis.[57] This theory was supported by Sadeghi et al[58] who based the highest antibacterial activity of silver nano-plate NPs compared to nano-spheres and nano-rods on the larger surface area that binds with the bacterial cells, as well as by Hao et al[53] who proved that mesoporous silica long-rod NPs had higher internalization and retention than spheres and short rods. In addition, various nano-shaped particles have also been shown to have diverse accumulation capabilities in different organ systems. Decuzzi et al[59] identified the shape

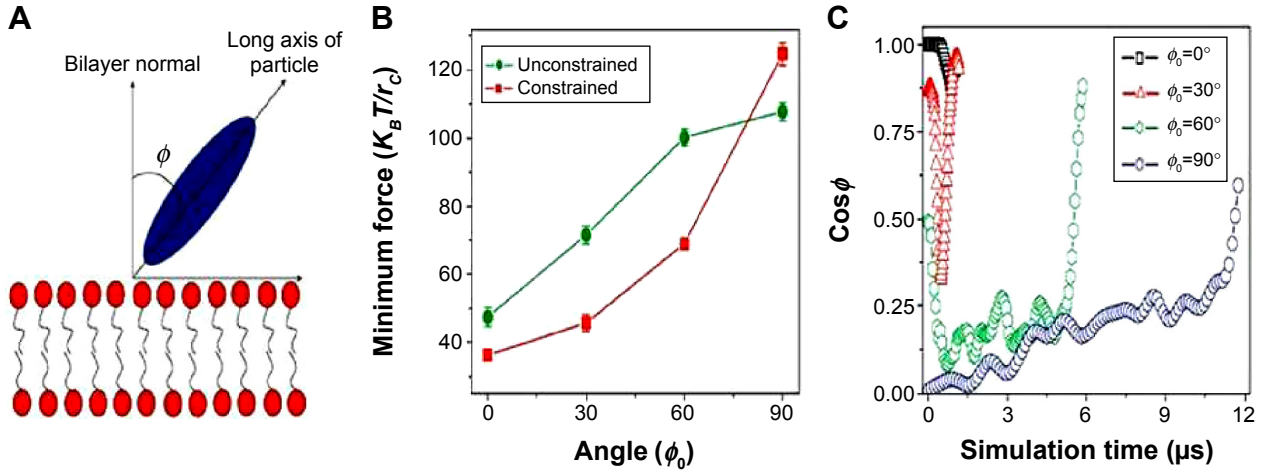

Figure 6 Particle internalization based on orientation to the membrane.
Notes: (**A**) Schematic showing the angle between the long axis of the particle and the bilayer normal; (**B**) minimum driving forces required to guide the ellipsoid with different initial orientations of the long axis through the lipid bilayer; (**C**) time evolution of particle orientations during the ellipsoid penetration processes with different initial orientations. Reprinted by permission from Macmillan Publishers Ltd: *Nature Nanotechnology*. Yang K, Ma YQ. Computer simulation of the translocation of nanoparticles with different shapes across a lipid bilayer. *Nat Nanotechnol*. 2010;5(8):579–583. Copyright © 2010.[56]

effect of silicon NPs and their accumulation in specific tissues, which they evaluated from their biodistribution data, that in the lung discoidal-shaped NPs tended to internalize more than spherical, cylindrical, and quasi-hemispherical NPs. In the liver, cylindrical NPs accumulated more than the other three shapes, discoidal-shaped NPs accumulated most in the heart, and discoidal and quasi-hemispherical NPs had the highest internalization in the spleen tissue. These results were further corroborated by Park et al,[60] who demonstrated that nano-worms had a higher tumor uptake in a fibrosarcoma and breast cancer cell line compared with spherical NPs, and Devarajan et al[61] who showed preferential accumulation of irregular spherical NPs in the spleen, while regular spherical NPs accumulated in the liver.

Contrary to the abovementioned observations that promote nonspherical NPs as possessing higher internalization, we cannot deduce that spherical NPs are less conducive for internalization, as several studies attest to their dynamic characteristics and unmatched high surface area to volume ratio. In fact, if considering therapeutic potential of NPs, then spheres should be superior to nonspherical NPs due to their excellent drug-loading capacity. Many researchers claim hypothetical theories on the internalization kinetics based on NP shape; however, we cannot deny that all of these NPs investigated do not have constant additional NP parameters, and these would also impact on the rate of cellular uptake.

Surface properties

The surface properties of NPs are as fundamental as the other key characteristics that dictate internalization. For targeted drug delivery, high circulation time of NPs in the body is required for the NPs to recognize their specific site of interest. Opsonins adsorbing to the surface of hydrophobic NPs decrease circulation time by initiating the immune response cascade which allows phagocytosis of the NPs following recognition as foreign objects. If the drug is unnecessarily taken up by the reticuloendothelial system, drug bioavailability is reduced and undesirable effects are exerted on the immune system and pose the threat of toxicity within the host.[62,63] NP hydrophobicity may instigate redundant interaction with plasma proteins, phagocytic internalization, immune cell stimulation and particle clearance.[64] Minimizing the recognition of NPs by the reticuloendothelial system and subsequent immune system will enhance the probability of uptake by the target cells. Hence, recent research is focused on modifying conventional hydrophobic NP surfaces with a hydrophilic protective layer. This layer creates a cloud of chains at the NP surface to cause steric repulsive forces against plasma proteins and increase the blood circulation half-life of targeted nanocarriers as shown in Figure 7.[24,65–67] Hydrophilic-coated NPs can be fabricated by making use of polymer types such as PEG, PEG-based copolymers and poly-vinyl pyyrolidone (PVP).[65,68]

Physicochemical surface parameters can affect cellular uptake as reported in the recent study conducted by Loh et al.[42] The study ascertained the theory of concentration-dependent chitosan molecules increasing NP transport through membranes by disrupting the integrity of intercellular TJs involved in paracellular transport.[69] Chitosan redistributes cytoskeleton proteins such as actin and tubulin found in the apical membrane resulting in the opening of TJs.[70] Using chitosan-coating as a surface modification for NPs could optimize paracellular transport and deliver a higher amount of drug-loaded nanocarriers to the targeted site.

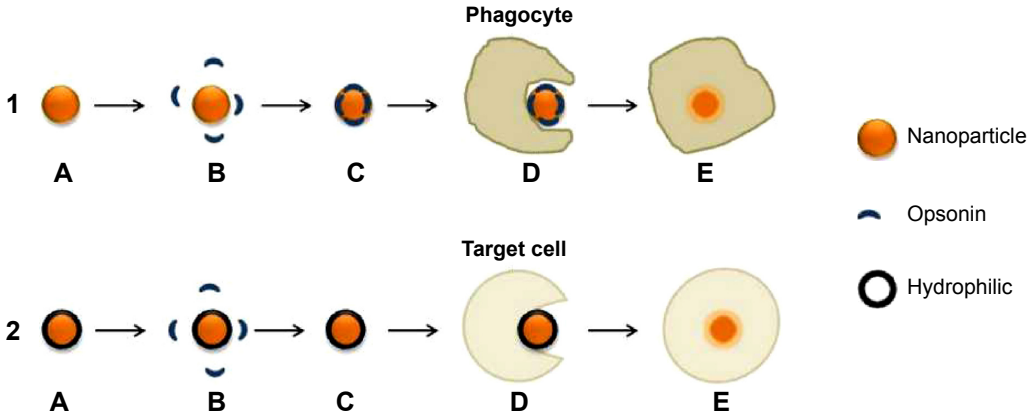

Figure 7 (**1**) Pathway of uncoated hydrophobic nanoparticle; (**2**) pathway of coated hydrophilic nanoparticle.
Notes: (**1**) (**A**) Nanoparticle in blood circulation; (**B**) opsonins recognize nanoparticle as a foreign body due to the hydrophobic surface; (**C**) opsonization of nanoparticle; (**D**) and (**E**) phagocytosis by phagocyte and elimination of nanoparticle. (**2**) (**A**) Hydrophilic polymer-coated nanoparticle in blood circulation; (**B**) steric hindrance maintains repulsive forces between opsonins and nanoparticle; (**C**) nanoparticle continues to circulate until target site reached; (**D**) and (**E**) endocytosis by target cell.

An interesting recent advance in research shows the coating of a polymeric NP core with red blood cell (RBC) membranes. Luk et al[71] synthesized a cloaked NP utilizing the RBC membranes to evade the immune response cascade resulting in a prolonged in vivo circulation time. This bio-inspired nano-system enhances the probability of NP uptake and if coupled with other NP factors to promote increased cellular uptake may prove to be yet another breakthrough drug delivery system. Therefore, modulating surface characteristics can control internalization rate, extent and even transport pathways. However, in order to achieve the desired outcome, the selection as well as manipulation of appropriate biomaterials for coating is imperative.

Proteins and ligand attachments

Cell-penetrating proteins or peptides (CPPs) can increase cellular uptake of surface-modified drug-loaded NPs by employing direct cell penetration or receptor-mediated endocytic pathways and localizing NPs at the required site.[72,73] CPPs are small amphipathic or cationic polypeptides (10–30 amino acids long) inclusive of trans-activating (TAT) peptide, penetratin, transportan, toxins, poly-arginine, and rabies virus glycoprotein (RVG).[74,75] A possible mechanism of the TAT protein is that it binds to cell surface heparin sulfate proteoglycans and is then internalized through receptor-mediated uptake (Figure 8).

Bareford and Swaan[76] reviewed cell adhesion molecules (CAMs), which form part of the subfamily of immunoglobulins. CAMs bind to cell adhesion receptors (CARs) on cell surfaces and stimulate clathrin-mediated uptake. Peptides such as Arg-Gly-Asp (RGD) bind to CARs and have been used extensively to promote the uptake of drug-loaded NPs. Furthermore, cationic proteins have a higher affinity to negatively charged cell membranes, which further promotes cellular internalization.[33,76] Ligands for enhanced cellular uptake include folic acid, albumin, and cholesterol, which are internalized using caveolae-mediated uptake, and are common, attractive methods to stimulate spontaneous uptake through receptors.[76] In contrast, Pujals and Giralt[77] reviewed the highly improved internalization efficiency of fatty acids or silaproline, which is a hydrophobic derivative of proline-rich, amphipathic CPPs. Targeting tissue-specific receptors and molecules by using antibodies has also proven to be useful for enabling internalization of drug-carriers intended for site-specific delivery. This strategy requires the conjugation of receptor-specific ligands and proteins to the NP coat. The potential for binding to the target tissue is dependent on the abundance of the ligand and its specificity and affinity for binding to the target cell membrane.[3]

By targeting the monoclonal antibody to the protein aminopeptidase P (APP) in the lung, in vivo transport across the endothelial barrier to lung tissue occurs within seconds.[78] NPs that are surface modified with cyclic RGD peptides preferably bind to $\alpha_v\beta_3$ integrin receptors. In comparison to unmodified NPs, these peptide functionalized NPs internalize more readily into HeLa cells.[79] This concept can be applied to tumor cells that contain specific membrane antigens where the surface of the drug carrier is modified with the corresponding antibody for increased binding and uptake. This is experimentally shown by targeting prostate-specific membrane antigen,[80] transferrin-conjugated NPs that demonstrated higher cellular uptake in a prostate cancer cell line,[81] and monoclonal antibody–conjugated PLGA NPs for increased tumor cell uptake.[82] In contrast, quantitative and biodistributive data from the study conducted by Huang et al[83] showed that three different ligands (single-chain variable fragment, amino terminal fragment, and cyclic RGD peptide) only marginally improved gold NP accumulation in tumor tissue in comparison with nontargeted controls.[83] Similarly, Temsamani and Vidal[84] reviewed the construct of phosphopeptides linked with penetratin as having inhibitory effects on ligand-dependent transduction pathways in various cell lines,

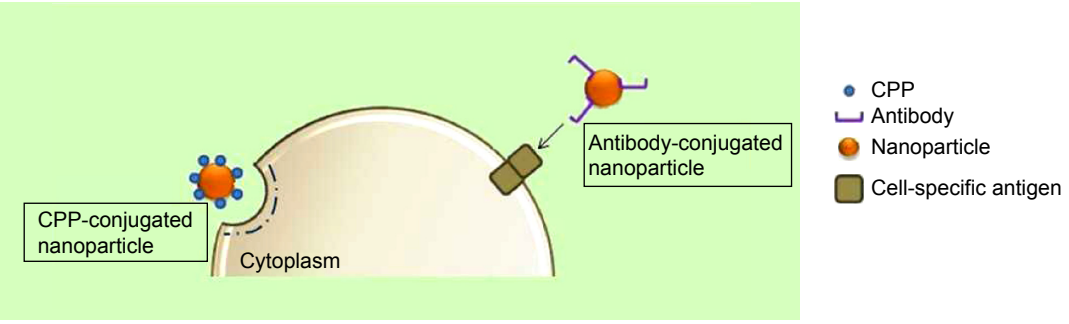

Figure 8 Stimulating endocytosis through CPP and antibody conjugation of nanoparticles.
Abbreviation: CPP, cell-penetrating protein or peptide.

even though individually, these CPPs promote internalization. The use of serum protein attachments on gold NPs was shown to improve their uptake half-life, rate, and extent.[36] α- and β-globulin proteins are known to be internalized by cells and increasing the diversity of protein attachments may allow entrance into cells via the receptor-mediated pathways. However, another study reported that serum protein attachments inhibited the uptake of polyvalent gold NPs, and that instead, uptake is dependent on scavenger receptors.[85]

Taking note of the use of a cationic mixed monolayer of CPPs and PEG, Liu et al[86] proved its efficiency on internalization rates of gold NPs by combining these factors. The multifunctional NPs show superior uptake when compared to NPs synthesized exclusive of the addition of CPP or having an anionic surface charge. Thus, drug delivery scientists have capitalized on the knowledge of receptors and ligands as targeting moieties for specific cellular organelle targeting.[76,87] This principle is not only applied to general cells but can also be adopted for specific targeting to tumors for increasing the bioavailability of drugs to the target site.

Current advances in trans-barrier internalization

It has been discussed how the inherent physical and chemical properties of NPs such as size, shape, surface charge, solubility, surface characteristics, and ligand complexes can dictate the degree of biocompatibility and internalization kinetics for NPs, as well as the selectivity of these factors for specific cell types. The additional key parameter for consideration in the uptake of NPs is the environment the nanosystem comes into contact with[29] and the manner in which we can engineer these parameters to trigger different biological responses.[69] As discussed by Brannon-Peppas and Blanchette,[88] the size of NPs for crossing biological barriers is dependent on the tissue, target site, and circulation. Likewise, all other NP characteristics need to be fabricated in consideration of inherent target tissue requirements for cellular internalization. Barrier capacity and trans-compartment transport of particles vary considerably between different tissue types.[89] Limited transport across the epithelia is one of the prime obstacles for therapeutic agents and nanomedicines reaching the adequate biological compartment,[1] as barrier systems are unable to evaluate and differentiate drug delivery systems for translocation from foreign particles, and therefore restrict the entry of the drug carriers, rendering the system invaluable and reducing its efficacy.

The delivery of therapeutic agents requires successful negotiation of these barriers in order to attain a sufficient therapeutic index.[90] To transverse these barriers using smart nanosystems, the development of efficient nanomedicines requires a thorough understanding of the characteristics of the body's systems, biological barriers, and mechanisms to evade foreign particle interactions in the body. Once the characteristics of the biological systems are defined, we can identify NP parameters that enhance transmembrane transport or cellular uptake (Table 2). From the data reported in Table 2, it is evident that various nanosystems contributing different parameters and characteristics are able to transverse biological barriers dictated by the barrier's set of limitations and specific NP criteria for internalization.

Conclusion

The scope for further research into this concept has immense potential to progress medical care by decreasing side effect profiles due to specific and targeted drug release, improving bioavailability, and bypassing first-pass metabolism. The use of sophisticated nanomedicines as effective vectors of drugs exerts beneficial effects at the molecular level, providing targeted drug delivery. The forefront of nanomedicine research comprises diagnosis, treatment, monitoring, and management of biological conditions. Independently, nanotechnology has surfaced as one of the most successful drug delivery concepts to date, and it is obvious that applications for biomedical nanotechnology are broad. This review encompasses possible techniques of NP manipulation to further improve intracellular delivery of drugs. The benefits of enhanced cellular internalization have been extensively motivated and the scope for further research in this domain is constant. Therapeutic advantages can be demonstrated by increasing NP cellular uptake, targeting site-specific organ systems by increasing selectivity, and even altering the drug release kinetics of the nanosystem and biodistribution. Taking this concept into consideration also allows for a single NP of ideal characteristics to encapsulate several drugs for delivery to enhance efficacy and possibly reduce resistance. Furthermore, the diversity of NP parameters with regards to targeting ligands can be studied thoroughly to assist with the internalization of macromolecule drugs that primarily have difficulty penetrating the cell membrane.

Apart from cellular internalization, focus has been placed on nuclear targeting via a nuclear localization signal (NLS).[143–145] At a complex level, drug delivery targeting intracellular organelles postcellular internalization may prove to be a research area of high interest. Included in this paradigm is the possibility of the multistage drug targeting of NPs from bypassing the cellular membrane

Table 2 Overview of biological barriers and nanoparticle advances to overcome physiological limitations

Organ system	Barriers to internalization	Physicochemical nanoparticle modification	(Trans)-epithelial transport mechanisms	Reference
Skin	Insoluble corneocytes and tight junctions in viable epidermis	• Chemical enhancers (oleic acid, ethanol, PEG) to surface coat	Pores, trans/intercellular, follicular penetration	91,92
		• 215.2 nm anionic quercetin-loaded lipid NPs	Intercellular permeation	93
	Pilosebaceous (10–70 mm) and sweat glands (60–80 mm)	• <10 nm metal maghemite NPs	Lipidic matrix, follicular penetration	94
	Intercellular lipidic matrix	• Hydrophilic 40 nm irregular, spherical PEG-b-copolymer NPs	Follicular penetration	94,95
		• 18 nm hydrophobic cationic/neutral 18 nm ellipsoid/spherical quantum dots	Pores, follicular penetration	96
Blood	Complement system, phagocytosis	• Heparin-complexed cerium oxide NPs for monocyte drug delivery		97,98
	White blood cells	• Cell membranes contain regions of +ve and –ve charge, NPs of either charge can be internalized		98
Ocular	Blood–aqueous barrier	• 180 nm anionic sparfloxacin-loaded PLGA NPs	Nasolacrimal drainage system	99,100
	Blood–retinal barrier	• 3.46 μm surfactant-complexed multilamellar acetazolamide niosomes		100,101
	Precorneal tear film (3–10 μm)	• 161 nm PEG-coated poly-ε-caprolactone nanocapsules	Transcytosis	102
Spinal cord	BSCB	• 2–5 nm cerium oxide NPs		103,104
	Astrocytic foot processes	• Ideal properties for penetrating BSCB/astrocytic foot processes		103
		• NP size (<50 nm), cationic, hydrophilic, CPP-complexed		12,36 45,67,74
Brain	BBB, blood–cerebrospinal fluid barrier	• Hydrophilic PEG-coated poly hexadecyl cyanoacrylate	Caveolae-mediated endocytosis	105,106
		• Chemical/biological/physical modulators for opening BBB	Trans/paracellular	107,108
	Enzymatic BBB	• MAb 5C6, to CR3 receptor-a β_2-integrin present on microglia	Receptor-mediated transcytosis	109
		• RMP-7 (bradykinin analog)-coated 50 nm NPs		110
		• Polyether-copolyester dendrimers	Clathrin/caveolae-mediated uptake	111
	High transendothelial electrical resistance of 1,500–2,000 V/cm^2	• Cationization of antibodies to undergo active transport	Absorptive mediated transcytosis	109
		• Anionic, 90 nm transferrin (Tf)-conjugated polymersomes	Tf receptor-mediated transcytosis	112
		• Anionic, ≤200 nm SLNs		113
		• Cationic, 190–210 nm nano-lipid emulsion	Caveolae-mediated end/macropinocytosis	114
	Brain extracellular space of 38–64 nm	• Anionic, 154 nm PLGA NPs via inner ear administration	Pinocytosis	115,116
		• 80 nm TiO_2 NPs administered through intranasal instillation	Transcytosis	117
Liver	Tight junctions, 5–10 μm wide BVs	• Filo-shaped micelles with high aspect ratios and cylindrical shape	Clathrin/caveolae-mediated endocytosis	59,118,119
		• 20 nm carboxylated polystyrene		120
		• 47.2 nm cationic chitosan NPs of irregular shape modified with glycyrrhizin complexation	Receptor-mediated endocytosis	121
	Phagocytic properties of juxtaposed Kupffer cells	• CPPs conjugated on <90 nm cationic NPs to target AGP receptors on hepatocytes for direct drug delivery	Receptor-mediated endocytosis	122

(*Continued*)

Table 2 (*Continued*)

Organ system	Barriers to internalization	Physicochemical nanoparticle modification	(Trans)-epithelial transport mechanisms	Reference
		• Saturating Kupffer cells with excess of drug-loaded NPs		119
		• Particles of 1–3 μm evade ruffled surface of Kupffer cells		123
	Protective mucin layer	• Neutral, hydrophobic NPs <200 nm		124
Oral cavity	Non-keratinized epithelia, saliva mucus, membrane-coating granules of buccal mucosa	• NPs entrapped within solid lozenges, chewing gum, flexible adhesive patches, and viscous liquids to coat mucosa		125,126
GIT	Tight junction barriers, cell composition	• Coating drug-loaded NPs with bacterial invasive ligands to target M cell surface components	Receptor-mediated endocytosis	91,127
	Low pH gradients	• pH-sensitive cationic 343 nm trimethylchitosan and 212 nm PLGA-PEG mannose NPs	Receptor-mediated endocytosis	90,128
	Thick, anionic mucus layer	• 154 nm cationic poly-6-cationic amphiphilic cyclodextrin–DNA complex internalized by intestinal epithelial cells	Macropinocytosis	129,130
	Macrophages	• Hydrophobic, neutral aminated NPs	Transcytosis	131
Lungs	Alveolar–capillary barrier, complex tight junctions	• 15–100 nm gold NPs	Transcytosis	132
		• Commercial multi-walled carbon-NTs disrupt tight junctions	Paracellular	133
		• Hydrophilic, surfactant-coated, enzymatic 20 nm PEI-PLGA NPs in 1.6 μm microgel		134
		• Discoidal-shaped NPs		59
	Alveolar lining fluid	• 200 nm NPs complexed with Fc portion of IgG	Receptor-mediated endocytosis	135
	Macrophages	• 235 nm protein-based NPs (serum albumin and transferrin)	Receptor-mediated transcytosis	4,134
Kidney	200–300 nm thick glycocalyx layer	• 78–100 nm spherical PLGA NPs	Caveolae-mediated endocytosis	136,137
	Phagocytic mesangial cells	• Albumin/streptavidin as ligands targeting renal tubular cells	Receptor-mediated endocytosis	138,139
	Cationic decomplexation	• Anionic derivatives carboxylated, co-dimethyl maleic acid, acetylated low-molecular weight chitosan, or PVP as NP coating	Receptor-mediated endocytosis	140,141 142

Abbreviations: AGP, alpha 1-acid glycoprotein; BBB, blood–brain barrier; BSCB, blood–spinal cord barrier; BV, blood vessel; CPPs, cell-penetrating proteins or peptides; GIT, gastrointestinal tract; NP, nanoparticle; NT, nanotube; PEG, poly(ethylene glycol); PEI, polyethylenimine; PLGA, poly(lactic-co-glycolic acid); PVP, poly-vinyl pyyrolidone; SLN, solid lipid nanoparticle.

to the organelle level of therapeutics. By combining many physicochemical and mechanical NP parameters specific to certain cell types or organ systems, an optimal level of cellular uptake can be achieved. Research has specified several NP characteristics that are pertinent for efficient internalization; however, it is immensely limited on more detailed trans-barrier uptake kinetics that would crucially improve NP efficacy and intracellular targeting while maintaining stability and nontoxicity. Nano drug carriers can be greatly exploited provided ex vivo and in vivo research is extensively conducted.

Pertinent to the abovementioned NP factors, we need to understand that by optimizing NP parameters and characteristics that support internalization theories and studies for enhanced intracellular delivery, these designed systems may not promote optimal drug entrapment or have adequate drug release. The issue of drug delivery design can be addressed but other in vivo requirements such as adequate clearance from systemic circulation, release of drugs from nontargeted sites, drug release from the nanosystem, and elimination of the nanocarrier from the body need to be adhered to. As the knowledge of physicochemical and physiological in vivo

processes improves, nanomedicine can be further specialized to attain the absolute effect intended.

Disclosure

The authors report no conflicts of interest in this work.

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