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Topical Review

Understanding cellular interactions with nanomaterials: towards a rational design of medical nanodevices

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Abstract

Biomedical applications increasingly require fully characterized new nanomaterials. There is strong evidence showing that nanomaterials not only interact with cells passively but also actively, mediating essential molecular processes for the regulation of cellular functions, but we are only starting to understand the mechanisms of those interactions. Systematic studies about cell behavior as a response to specific nanoparticle properties are scarce in the literature even when they are necessary for the rational design of medical nanodevices. Information in the literature shows that the physicochemical properties determine the bioactivity, biocompatibility, and safety of nanomaterials. The information available regarding the interaction and responses of cells to nanomaterials has not been analyzed and discussed in a single document. Hence, in this review, we present the latest advances about cellular responses to nanomaterials and integrate the available information into concrete considerations for the development of innovative, efficient, specific and, more importantly, safe biomedical nanodevices. We focus on how physicochemical nanoparticle properties (size, chemical surface, shape, charge, and topography) influence cell behavior in a first attempt to provide a practical guide for designing medical nanodevices, avoiding common experimental omissions that may lead to data misinterpretation. Finally, we emphasize the importance of the systematic study of nano–bio interactions to acquire sufficient reproducible information that allows accurate control of cell behavior based on tuning of nanomaterial properties. This information is useful to guide the design of specific nanodevices and nanomaterials to elicit desired cell responses, like targeting, drug delivery, cell attachment, differentiation, etc, or to avoid undesired side effects.

Keywords: nanoparticle, nanomaterial, size, shape, nano device, protein corona, nanoparticle coating

(Some figures may appear in colour only in the online journal)

Abbreviations

CNT	carbon nanotubes
FA	focal adhesion
hADMSC	human adipose-derived mesenchymal stem cells
HAPNs	hydroxyapatite NP

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hMSCs	human bone marrow derived mesenchymal stem cells
hESC	human embryonic stem cells
IC50	half maximal inhibitory concentration
MW	multiple walled
NP	nanoparticle
PEG	polyethylene glycol
PDMS	polydimethylsiloxane
RME	receptor-mediated endocytocis
ROS	reactive oxygen species
SW	single wall

1. Introduction

The development of nanomaterials has created a new set of tools with unforeseen applications. Their size (1–100 nm) is in the same order of magnitude as cellular organelles, allowing a direct interaction of nanomaterials with cells, making them unique tools for influencing biological pathways and processes. Such interactions open a whole range of possible applications in biology (Nel *et al* 2009). It has been widely demonstrated that an active interaction between living organisms and nanomaterials exists and that it impacts cell physiology, eliciting both positive and negative responses. For example, while carbon nanotubes (CNT) promote differentiation of human embryonic stem cells into neurons (Chao *et al* 2009), they also induce oxidative stress, membrane damage, and cell death in transformed murine macrophages RAW 264.7 (Khaliullin *et al* 2015, Shvedova *et al* 2015). Both favorable and deleterious responses are not fully understood, but it has been shown that the physicochemical properties of CNT affect such interactions (Jiang *et al* 2008, Nel *et al* 2009, Wang *et al* 2018). As the potential of nanomaterial and cellular interactions is more extensively recognized, new materials are being designed with the specific purpose of interacting with cells. The wide variety and nature of such materials and the possible range of cell types and responses make it necessary to summarize the current knowledge of nanomaterial–cell interactions. An analysis of the state of the art is necessary for the rational design of novel, useful, and safe nanomaterials with applications in medicine.

Gold nanoparticles (AuNP) are the most widely used nanomaterial for medical applications due to their chemical stability, easy modification of their surface chemistry, and their tunable optical properties. AuNP have been exploited for nanobiotechnological applications for the last two decades, and they play essential roles in the fabrication of nanomedicine tools. In addition, AuNP are easily functionalized and can be used as drug carriers when functionalized with chemical groups that target specific cells. Due to the high electron density of Au, they have been extensively used for enhanced imaging diagnosis (Ning *et al* 2017, Chugh *et al* 2018, Kalimuthu *et al* 2018). Because most of the available information in the literature is about gold NP, many of the papers discussed here refer to them. Nevertheless, nanoparticles of other materials are gaining increasing importance, and are also discussed when relevant.

In this review, we summarize the efforts, advances, and limitations of the current knowledge about cellular responses to nanomaterials. This work pretends to be useful to guide the design of nanodevices that influence cells by specific targeting, drug delivery, cell attachment promotion, differentiation, etc, while avoiding undesired side effects, such as oxidative stress, cell death, and unspecific interactions. Also, we simplified complex mathematical models to predict nanoparticle (NP) uptake, a practical parameter in NP design that could be of interest to non-mathematical researchers.

2. Size matters

Nanostructured materials of various shapes and sizes interact with cells in different ways. Most studies regarding the effect of the size of nanomaterials have been performed with spherical NP, which are synthesized from the bottom-up, and are the main topic of discussion in this section. However, it should be considered that the size of one- or two-dimensional materials can also have critical cellular interactions. Nanomaterials with non-spherical shapes are discussed in section 3.

A particular challenge of determining the effect of NP size on the interactions with cells is that methods to measure size remain to be standardized. Different techniques have been used to measure NP size, each with intrinsic limitations. However, for relevant size determination, NP should be resuspended and measured in the same medium/solvent that will be used in the biological experiment or context. Solvents have different ionic strengths and pH, and influence NP surface charge, aggregation, stability, but especially the protein corona formed over the NP surface. Recently, it was published that a protein corona formed on NP in contact with serum significantly enlarges the NP hydrodynamic radius *in vivo* (Wang *et al* 2017, Garcia-Álvarez *et al* 2018). Typically, incubation of nanomaterials with cells in a culture medium results in adsorption of serum proteins on their surface (Verma and Stellacci 2010), and it has been reported that protein corona modifies NP aggregation and size. The most frequent proteins involved in protein corona formation are globular albumins, fibronectin, complement proteins, fibrinogen, immunoglobulins, and apolipoproteins (Cedervall *et al* 2007, Lundqvist *et al* 2008). Also, protein corona can provide undesired cell effects, because the NP surface properties can be masked for the protein corona and this undesired coating can complicate the relationship between NP chemical functionality and their biological effects. Recently it was demonstrated that targeting capacity of ligand-modified NP was lost after incubation with plasma *in vitro* (Zhang *et al* 2018). This fact has been widely neglected in the literature, possibly leading to incorrect or imprecise conclusions about how NP size influences cellular responses. This should be considered while reading the effects detailed below.

2.1. Size-dependent toxicity

For a long time, AuNP have been considered as nontoxic, but recently AuNP size-dependent toxicity has been detected. It is worrisome that the safety of AuNP has been mostly taken for granted despite the poor understanding of their effect on cellular physiology. NP with diameters between 1 and 100 nm have been found to alter processes essential for basic cellular functions, including active and passive cell death (Jiang *et al* 2008). The smallest nanoparticles, with diameters below 2 nm, have been identified in most papers as toxic to cells. Pan *et al* (2007) observed that AuNP of 1.4 nm caused the death of different cell types (connective tissue fibroblasts, epithelial cells, macrophages, and melanoma cells), with IC50 values from 30 to 56 μM. In contrast, in the same study, the authors observed that AuNP of 15 nm of diameter were nontoxic even at concentrations up to 100 fold higher (Pan *et al* 2007). Similar results have been reported by Tsoli *et al* (2005), who showed that Au55 clusters of 1.4 nm capped with triphenylphosphine monosulfonate cause death of metastatic melanoma (MV3) and nonmalignant cell lines. Three mechanisms have been proposed to explain the toxicity of Au55 clusters: the first, their perfect fit in the major grooves of DNA, the second, increased ROS production and the third, blockage of membrane ion channels (Tsoli *et al* 2005, Pan *et al* 2009, Leifert *et al* 2013).

Sublethal effects upon exposure of nanomaterials are also related to size. Senut *et al* (2016) studied the effect of AuNP of different core sizes (1.5, 4, and 14 nm) on the viability, pluripotency, neuronal differentiation, and DNA methylation of human embryonic stem cells (hESC). The smallest AuNP were toxic to hESCs, probably due to their interference with membrane functions, as well as their larger surface area-to-volume ratio. Interestingly, AuNP of 4 nm caused a decrease of more of 20% of DNA methylation in hESC, while no toxicity was observed. Otherwise, AuNP (14 nm) and AuNP (4 nm) did not induce alterations in the differentiation potential of hESCs at doses of 10 μg ml^{-1}, after two weeks of exposure. Also, AuNP of 4 nm or 14 nm do not alter the neuronal differentiation capabilities of hESCs (Senut *et al* 2016). Size-dependent toxicity has also been reported for other NP. For example, the cytotoxic effects of silver nanoparticles (AgNP) of various sizes (10, 40, and 100 nm) were tested on reproductive, pulmonary and bone cells (CHO-9, the Sertori cell line 15P-1, RAW264.7 and MG-63 cells). All AgNP were toxic, but the most toxic were the smallest (10 nm) (Zapór 2016, Xie *et al* 2019). These results agree with Kim *et al* (2012), who observed that AgNP (10 nm) have a higher ability to induce apoptosis in osteoblast precursor cells MC3T3-E1 than larger AgNP (Kim *et al* 2012).

It is necessary to glance at NP uptake mechanisms to understand how NP are incorporated into cells and NP size dependent toxicity. We will refer to this in the next section.

2.2. Size-dependent NP uptake

The 'perfect' size for nanomaterials to be used for drug delivery or cancer therapy has been the subject of recent discussion. Available evidence shows that there is not a universal size for optimal NP internalization, as the optimal size is different for each cell type. Nevertheless, most of the literature has proposed 50 nm as an adequate size for the efficient internalization of NP, based on diverse experiments involving hydroxyapatite NP (HAPNs) (45 nm), selenium NP, other AuNP (50 nm) and polypyrrole nanoparticles (60 nm) (Yuan *et al* 2010, Ma *et al* 2011, Cui *et al* 2019). An example of how this knowledge can be applied to a better design of therapeutic nanomaterials is the size-dependent antitumor activity of HAPNs in human hepatoma cells HepG2, which had an uptake dependent of size in the following order: 45 nm $>$26 nm $>$78 nm $>$175 nm. HAPNs ranging from 20 nm to 80 nm effectively activated caspase-3 and caspase-9, decreased the Bcl-2 protein level, and increased the levels of Bax, Bid, and the release of cytochrome c from mitochondria, with 45 nm HAPNs as the most efficiently internalized.

Cellular plasma membranes are dynamic and selectively permeable fluids that not only delimit the cellular perimeter but also control trafficking into the cell. A size-dependent interaction exists between the NP and the required signaling for its uptake by cells. Mathematical modeling has demonstrated that there is an optimal NP size for faster absorption and release from cells. This optimal size is a result of the competition between diffusion and thermodynamic driving force. NP with a small hydrodynamic radius have high diffusion constants but weaker interaction with cells. In contrast, larger NP have smaller diffusion constant but stronger interactions (Shi *et al* 2009). The model's prediction agrees with experimental data, including the uptake by various mammalian cell lines (CHO, cervical carcinoma epithelial HeLa, and MCF-7) of AuNP (20, 30, 50, and 80 nm) coated with a layer-by-layer approach with nucleic acids and poly (ethyleneimine). Reduced uptake was observed as NP size increased (Elbakry *et al* 2012). Also, a size-dependent accumulation of AuNP was observed in an *ex vivo* tumor model. Smaller AuNP (2–6 nm) were able to penetrate deeply into tumor spheroids, even reaching the cell nucleus, whereas 15 nm nanoparticles could not penetrate (Huang *et al* 2012). Chithrani *et al* (2006) studied the size-dependent AuNP intracellular uptake kinetics and saturation concentrations. They exposed HeLa cells to AuNP of three sizes: 14, 50 and 74 nm. The authors found that AuNP of 50 nm had the highest cell uptake, followed by AuNP of 14 nm, and finally, AuNP of 74 nm. Three types of AuNP were internalized via the receptor-mediated endocytosis pathway (RME). Authors speculated that nonspecific adsorption of serum proteins mediates the NP uptake half-life, rate, and amount of internalized AuNP (Chithrani *et al* 2006). In a later work, they studied the uptake of AuNP of the same sizes by MCF-7 and HeLa cells in normoxic and hypoxic conditions. Interestingly, they found that AuNP absorption was higher in hypoxic conditions, similar to those present in a tumor. These results highlight the AuNP potential to be used as a cancer therapeutic agent (Neshatian *et al* 2014). In addition, larger AuNP with a high surface area and strong absorption in the visible and near-infrared regions of the electromagnetic spectra make

anisotropic AuNP an excellent tool in hyperthermia and laser for cancer therapy (Jindal 2017).

In a systematic study, Shang *et al* (2014) evaluated the cellular uptake of a wider size range of AuNP (3.3–100 nm) by confocal disk microscopy. They found that a minimal quantity of small NP on the plasma membrane is required to initiate cellular internalization. NP with diameters of 10 nm accumulated on the plasma membrane before being internalized by cells. Thus, an individual small NP is not capable of triggering endocytosis by itself. In contrast, larger AuNP (≈100 nm) are immediately endocytosed without prior accumulation on the plasma membrane, independently of their surface charge. The authors discussed that this size-dependent uptake requires a sufficiently strong local interaction of the NP with the endocytic machinery to trigger subsequent internalization (Shang *et al* 2014). Interestingly, Cho *et al* (2011) showed that NP accumulation on the cell membrane and their consequent uptake is influenced by NP sedimentation. In that work, authors measured the number of AuNP internalized by cells in upright and inverted configurations. In the upright configuration, human breast cancer cells SK-BR-3 were cultured in a coverslip and placed at the bottom of the well, as is usually performed. In the inverted configuration, the coverslip was suspended above cells, facing upside down. The surrounding medium contained the NP. They observed that in the upright configuration, where NP settled because of gravity, NP uptake by cells was higher, due to the increment of the concentration of NP near the cell surface. The opposite effect was observed in the inverted configuration. It is worth mentioning that these results were independent of NP size, shape, density, and surface coating (Cho *et al* 2011). In contrast, Toy *et al* (2011) evaluated the effect of size (60–130 nm) and density (1–19 g ml^{-1}) of different types of NP (liposomes or metallic) in their margination on vessel walls on an *in vitro* model of microcirculation at a physiologically relevant flow rate. Interestingly, their results showed that NP density was more relevant than size (Toy *et al* 2011). Taken together, these reports invite researchers to be careful when interpreting their results while studying the influence of NP physical properties and their impact on cell behavior, and to exert caution when extrapolating *in vitro* results to *in vivo* models.

Not only does NP size matter, but also cell size plays an important role. In another interesting work, the role of the mechanical cell state in AuNP uptake was investigated. The size of human mesenchymal stem cells (hMSCs) was controlled by culturing them on micropatterned surfaces with microdots of different diameters. Then cells were exposed to AuNP (50 nm) capped with poly(ethylene glycol) (PEG) to improve biocompatibility and to avoid protein adsorption. Experimental data showed that larger cells had a higher total AuNP uptake, but lower uptake per cellular unit area, than smaller cells. The presence of two opposite effects can explain these observations. First, large cell sizes favor uptake due to the larger contact area with AuNP. However, larger cell sizes also result in an increment of membrane tension, requiring a high wrapping energy for engulfing AuNP and reducing uptake (Wang *et al* 2016). When a minimal quantity of NP accumulates in the cell periphery, NP–cell interactions modify the associated energy landscapes. For NP wrapping, it is necessary to curve the intrinsically curved cell membrane, which increases membrane tension. To overcome this tension, all the forces (electrostatic, van der Waals, hydrophobic interactions, ligand-receptor binding, etc) have a role in endocytosis.

2.3. Directed NP delivery: effect of size

NP–cell interaction can be directed to target cells by coating them with specific ligands. Experimental data have shown that NP size restricts the molecular dynamics of molecules attached to the NP surface, impacting ligand binding. This is particularly relevant for antibodies conjugated to NP. Jiang *et al* (2008) coated AuNP and AgNP (2–100 nm) with a monoclonal antibody (Herceptin) and evaluated their interaction with SK-BR-3 breast cancer cells. Results showed a significant increase of the uptake of NP coated with the antibody, in comparison with uncoated NP. Also, the authors found that NP with diameters between 40 and 50 nm were optimal for NP-antibody internalization, probably due to the balance between multivalent crosslinking of membrane receptors and the process of membrane wrapping involved in RME. The antibody density and the number of available Herceptin binding sites on the NP depend on the surface area. Thus, the higher surface area of smaller NP restricts the relative orientation between ligands and receptors. Moreover, the authors observed that the dissociation constant increased as NP size decreased. These observations are very important, because an early release of the NP-antibody complex can reduce its therapeutic function. Therefore, sufficiently big NP are needed to provide appropriate conformational freedom to the bound antibody, but not enough to cause early release (Jiang *et al* 2008).

The effect of size on directed NP delivery can be summarized as follows:

1) *Internalization rate:* specific interactions (ligand-receptor mediated) delay internalization, as wrapping requires that receptors diffuse to the binding sites. In contrast, in non-specific interactions, spontaneous binding elicits internalization driving forces when NP are close to the cell membrane (Gao *et al* 2005).
2) *Interferences with NP surface imperfections:* NP fractures or surface imperfections interfere with specific receptor recognition (Terdalkar *et al* 2010).
3) *Translational entropy increment:* Receptor density on NP remains statistically uniform to maximize entropy, but receptors can cluster if free energy is reduced. Thus, a high concentration of receptors has an entropic penalty (Freund and Lin 2004; Yuan and Zhang 2010). Related experimental data have demonstrated that smaller NP are wrapped faster, but require a higher ligand density to overcome the larger membrane bending energy needed for the higher curvature of smaller particles (Lane *et al* 2015).

How can we design an NP with the optimal size and number of ligands to obtain an efficient vehicle for cell targeting?

As a first approach to answer this question, we will consider the simplest case, a spherical NP with non-specific interactions with the cell membrane. In this case, the balance of adhesion energy and membrane deformation energy determines the lower NP radius ($R_{\min}$) that cannot be endocytosed (equation (1)) (Lane *et al* 2015). Mathematical procedures to obtain this and further equations can be consulted in the references.

$$R_{\min} = \sqrt{\frac{2B}{\alpha_{ns} - \sigma}}. \quad (1)$$

In equation (1), B is the membrane bending stiffness, α_{ns} is the adhesion strength and σ is the membrane tension. Notice that NP smaller than $R_{\min}$ can still enter cells by other pathways, for example, by simple diffusion.

Gao *et al* (2005) proposed a mathematical model for NP RME. Their model considers that there is a minimal particle radius (R_{th}) for endocytosis to occur (15 nm and 30 nm for cylindrical and spherical NP, respectively). It has been postulated that there is an optimal NP radius (R_{opt}) for endocytosis, associated with the shortest wrapping time (τ_w). However, this work does not consider non-specific interactions arising from electrostatic and osmotic (steric stabilization) forces, which play a fundamental role in cell adhesion (Bell *et al* 1984). In a further study, Decuzzi and Ferrari (2007) included both the influence of specific and non-specific interactions in receptor-mediated endocytosis of NP, obtaining two quantitative and more general expressions. The first one relates the minimum density of receptor-bound pairs for NP endocytosis and R_{th} (equation (2)) (Decuzzi and Ferrari 2007)

$$R_{th} = \sqrt{\frac{E}{2m_b C}}, \quad (2)$$

where E=bending energy factor = 20 (10–20); C=binding factor 15 (5–35). These values are reference data of the cell–particle system used in their numerical calculations. $m_b = \frac{m_r^0}{\tilde{m}}$, where m_r^0 is the receptor density at time zero (10^2 units/μm^2) and $\tilde{m}$ is the molecule density ratio (10^{-4}–10^{-1}). Physiological ranges of variation for the dimensionless governing parameters were used in the numerical calculations.

The next equations can be used to estimate the time required for the membrane wrap around the NP (equation (3)) (Decuzzi and Ferrari 2007)

$$\tau_w = \frac{1}{M}\left(\frac{\pi R}{2\alpha}\right)^2, \quad (3)$$

where τ_w is the wrapping time defined as the time needed to wrap half of the particle surface; R is the particle radius; α is a velocity factor and M is a mobility coefficient for cell receptors diffusing through the membrane (Decuzzi and Ferrari 2007). Both equations are very useful to guide in designing NP with controlled endocytic performances. Similar equations have been obtained to estimate the optimal radius of both viruses and NP to be uptaken via clathrin-mediated endocytosis, which is close to 60 nm (Banerjee *et al* 2016).

Mathematical models are of particular clinical significance because they provide information that can contribute to the rational design of more effective targeted therapeutic and diagnostic systems. However, the comparison of theoretical optimal size (and other NP sizes reported in the literature) for cellular uptake with experimental data should be performed carefully, because of the recent data that show that there are other parameters which influence NP size. One of them is the protein corona formed over NP at contact with serum, which significantly enlarges NP size (Garcia-Álvarez *et al* 2018). This fact has been ignored in most of the literature.

2.4. Size-dependent NP endocytosis pathways

Elucidation of the endocytic pathway involved in NP uptake is of crucial relevance for enhancing total NP uptake by cells, manipulating their intracellular trafficking and minimizing possible toxic effects (Huang *et al* 2002). Cells internalize molecules through various endocytic pathways. These pathways can be classified as specific and non-specific. Specific pathways include (a) Endocytosis clathrin- and caveolin-mediated: an energy-dependent process by which cells internalize molecules. (b) Phagocytosis (mannose receptor-, complement receptor-, Fcγ receptor-, and scavenger receptor-mediated). Phagocytosis is an actin-dependent endocytic process by which phagocytes engulf particles with sizes larger than 0.5 μm. Moreover, non-specific endocytosis pathways include (a) Macropinocytosis, a process by which cells internalize fluids and particles together, and large vesicles (0.2–5 μm) are formed. (b) Pinocytosis, a process by which cells absorb extracellular fluids, small molecules and small vesicles (≈100 nm) (Kou *et al* 2013). Each of these mechanisms depends on the cell type and differentiation state, but it has been recently demonstrated by the systematic addition of biochemical inhibitors that different pathways are also activated depending on NP size with which cells interact. Some of the most relevant works are summarized in table 1 and figure 1.

Taken together, results in table 1 highlight that the underlying mechanisms that mediate the internalization of non-targeted NP are not fully understood. In most cases, experimental data show that ≈100 nm sized NP are preferably internalized via clathrin-mediated endocytosis. The other endocytosis pathways used were highly dependent on the cell and material types, but it is possible to distinguish common NP uptake mechanisms, which are schematized in figure 1. Popp and Segatori (2019) studied the effect of zinc oxide particles and found that both NP and microparticles (100–1000 nm) induce autophagy, but only microparticles block the autophagic flux. It is worth highlighting that current knowledge suggests that NP size is not the only factor that determines the NP uptake mechanism, and that several endocytic pathways have a synergistic role in NP internalization. To understand how one endocytic pathway is

Table 1. Relevant studies of size-dependent NP endocytosis.

NP	Cell line	Endocytosis pathway	Additional observations	Reference
Au (13 and 45 nm)	Human dermal fibroblasts CF-31	45 nm: clathrin-mediated endocytosis. 13 nm: mostly phagocytosis.	AuNP cause reversible cytoskeleton filament disruption. Toxicity of different sized AuNP does not depend on total Au intracellular concentration.	Mironava *et al* (2010)
Au–cysteine conjugates labeled with amine-reactive Cy5 dye.	HeLa	4.5 nm: caveolae-mediated endocytosis.	Most NP were localized in intracellular endocytic vesicles in the perinuclear region.	Hao *et al* (2012)
Calcein-loaded $[Zr_6O_4(OH)_4]$ (UiO-66) with 1,4-benzenedicarboxylate (BDC) ligands (150 and 260 nm).	HeLa	150 nm: clathrin-mediated endocytosis. 260 nm: a combination of clathrin- and caveolae mediated endocytosis pathway.	Uptake mechanism of NP should be considered for the design of efficient drug delivery systems.	Orellana-Tavra *et al* (2016)
Carboxylated polystyrene (40 and 150 nm).	HeLa and MCF-7	40 nm: clathrin-mediated endocytosis pathway. 150 nm: caveolae-mediated endocytosis.	NP of 150 nm were preferentially in exosomes in comparison with NP of 40 nm, indicating that they were exocytosed.	Wang *et al* (2017a)
Fluorescent latex (500–1000 nm).	Non-phagocytic B16 cells (melanoma).	<200 nm: Internalization of microspheres involved clathrin-coated pits. 500 nm: caveolae-mediated endocytosis.	Rate of internalization of smaller NP was higher than that of larger NP.	Rejman *et al* (2004)
Green fluorescent non-functionalized polystyrene particles with diameters of 0.9, 1.9, 2.3, 3.0, 4.3, 5.7 and 9.0 μm.	Continuous alveolar rat macrophage cells NR8383, murine peritoneal macrophages J774 and human spleen macrophages.	Particles of 2–3 μm exhibited maximal phagocytosis and attachment.	Internalization rate not affected by particle size, but the internalization pathway was affected. Relevant for selecting the appropriate size for phagocytosis.	Champion *et al* (2008)
Poly-lactictide-co-polyethylene glycol (100 nm).	HeLa	100 nm: Clathrin-mediated endocytosis pathway.	Negatively charged NP entered the cells through a pathway different to endocytosis.	Harush-Frenkel *et al* (2007)
Cationic cross-linked poly(ethylene glycol) hydrogel. Cubic-shaped particles (cubic side length 2, 3 and 5 μm). Cylindrical-shaped particles with identical length and diameters of 0.5 μm or 1 μm. Cylindrical-shaped NP (diameters 200 nm, 100 nm, 150 nm).	HeLa	Clathrin-mediated and caveolae-mediated endocytosis and, to a lesser extent, macropinocytosis are involved with nano- and micro-particle internalization, but these mechanisms play a larger role in the internalization of smaller NP (150 nm and 200 nm).	NP charge relevant for cellular internalization. Positively charged NP were internalized in 84% of cells after 1 h, while the identical negatively charged particles were not significantly internalized.	Gratton *et al* (2008)
Mesoporous silica NP conjugated with fluorescein isothiocyanate (100 nm).	Human mesenchymal stem cells (hMSCs) and adipocytes (3T3-L1).	Clathrin-mediated endocytosis.	Inhibition of caveola-coated pit endocytosis by filipin did not affect NP uptake	Huang *et al* (2005)
Hydroxyapatite (20 nm, 80 nm and 12 μm).	Human umbilical vein endothelial cells (HUVECs).	20 and 80 nm: clathrin- and caveolin-mediated endocytosis. 12 μm: macropinocytosis.	Exposure to NP suppressed the angiogenic ability of HUVEC cells.	Shi *et al* (2017)
Oleoyl alginate ester.	Heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2)	50–120 nm: clathrin-mediated endocytosis. 420 nm: caveolae-mediated endocytosis. 730 nm: macropinocytosis.	Smaller NP showed the highest the cellular uptake and permeability.	Li *et al* (2015)

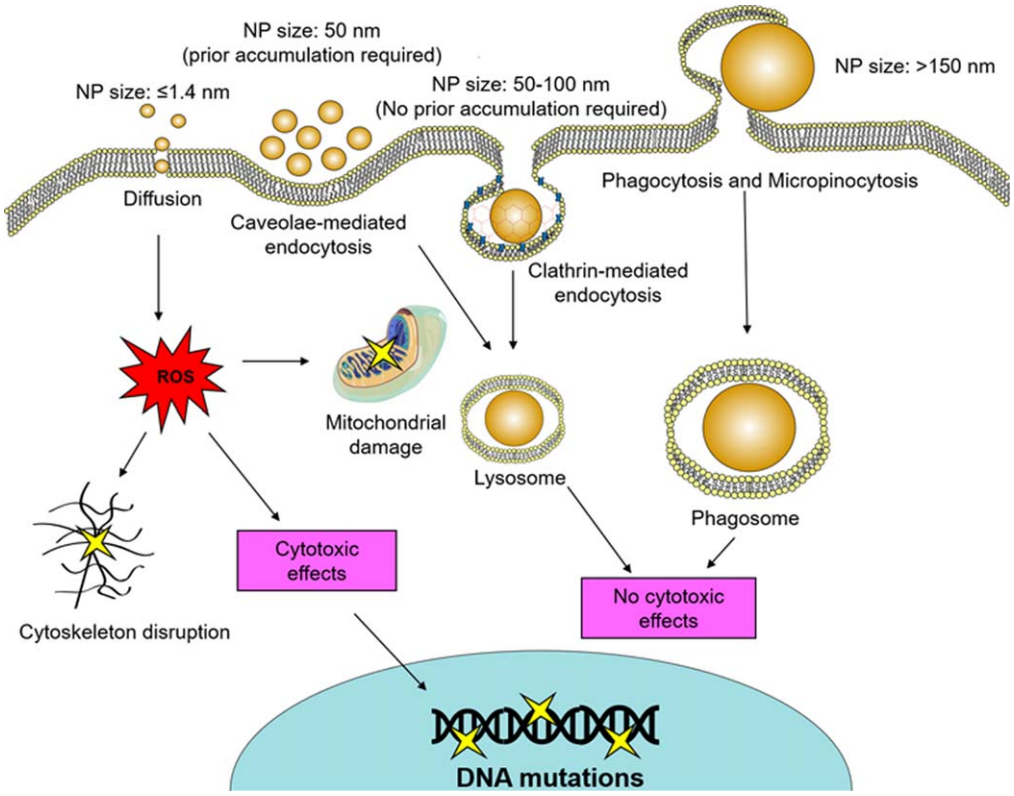

Figure 1. Size-dependent NP uptake mechanisms and cytotoxicity. The figure summarizes observations regarding the role of NP size on cellular internalization mechanisms. Small NP (⩽1.4 nm) have been typically reported as cytotoxic due to oxidative stress induction (ROS) that disrupts the cytoskeleton and damages mitochondria and DNA. NP with intermediate sized (≈50–100 nm) can be internalized by caveolae-mediated or clathrin-mediated endocytosis depending on the cell line and NP material, and finally, bigger NP (150–500 nm) can be internalized by macropinocytosis. In the case of intermediate- and big-sized Au nanospheres, no cytotoxic effects have been observed. It should be considered that both the NP material and the cell line influence internalization.

preferred over another, it is crucial to design efficient medical nanodevices to enhance the efficiency of internalization, accurate targeting, etc and ultimately to avoid undesirable side effects.

3. NP surface

The NP surface is the first part with which cells interact. Interactions cause intentional or unintentional biological effects due to NP charge, dipole–dipole interactions, van der Waals forces, solvation, electrostatic forces, solvophobic effects, depletion forces, etc (Nel *et al* 2009, Jing *et al* 2019). An excellent review was published by Makarucha *et al* (2011) about the use of computational techniques to study theoretical interactions between nanomaterials and biological systems (Makarucha *et al* 2011).

3.1. Electric charge

Electric charge plays an essential role in the NP adsorption and translocation across the cell membrane. *In silico* studies with positive- and negative-charged and uncharged NP interacting with an uncharged phospholipid bilayer have been performed by coarse-grained molecular dynamics. This data showed that electrostatic attraction improves the adhesion of charged NP to the membrane. An increasing electrostatic energy results in an almost complete wrapping of the charged NP. Adsorption of cationic NP induces local disordered transitions in the membrane, favoring an entropy increment. Meanwhile, negative-charged NP induced the formation of the highly ordered regions in fluid bilayers, which is entropically unfavorable. Thus NP internalization is driven by enthalpy (figure 2) (Li and Gu 2010). This agrees with experimental observations in red blood cells. Red blood cells do not have any phagocytic receptors on their membrane surface, and no actinmyosin system, so they are incapable of endocytosis. For this reason, it is a popular model to study the passive transit of particles across cell membranes. Using fluorescence microscopy, it has been observed that zwitterionic quantum dots penetrate through the membrane of red blood cells. Also, by infrared spectra analysis, a high lipid bilayer flexibility has been observed, while the membrane structure remained intact (Wang *et al* 2012). This strategy can be applied for designing medical nanodevices for direct drug delivery into the cytosol by passive NP uptake.

It has been found that positively charged AuNP attached to negatively-charged cell surfaces increase the cell membrane fluidity and the NP uptake rate. For example, positively charged hydrogel NP were internalized in 84% of HeLa cells after 1 h of incubation, while identically shaped and negatively charged AuNP were not significantly internalized

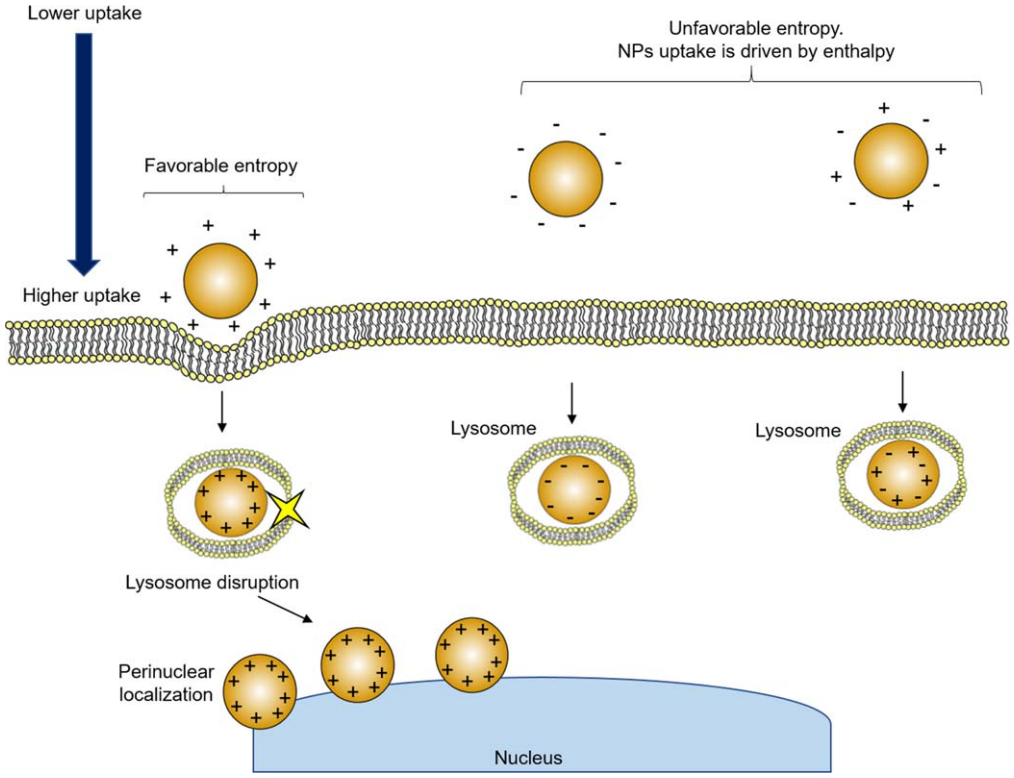

Figure 2. Surface charge-dependent NP uptake and cellular trafficking. Positively charged NP exhibited higher cell uptake. Adsorption of cationic NP induces local disordered transitions in the adhering region of the membrane, which increases entropy. Negatively and neutral-charged NP are poorly internalized. Negative-charged NP induced the formation of highly ordered membrane regions that are entropically unfavorable, but their entrance is driven by enthalpy.

(Gratton *et al* 2008). This is a result of the mechanisms that cells use to maintain the membrane charge, which include removal of attached AuNP through endocytosis or other pathways. During this process, the cell membrane probably loses its rigidity, and its morphology changes, increasing membrane permeability (Cho *et al* 2009). This could explain why the cellular uptake of various types of positively charged nanomaterials has resulted in higher uptake rates and efficiency in diverse cell types, in comparison with negatively charged NP (Wilhelm *et al* 2003, Yue *et al* 2011, Fröhlich 2012, Salatin *et al* 2015). However, coating NP with cationic ligands might not be a viable strategy to improve cellular uptake. There is experimental data that shows that cationic NP can disrupt the cellular membrane, resulting in a cytotoxic effect by changing the cell membrane potential and intracellular concentration of calcium ions (Nel *et al* 2009). Also, Yue *et al* (2011) synthesized chitosan-based NP with various surface charges, but keeping other characteristics identical, such as size, shape, matrix, and mechanical properties, to minimize the influence of other factors. They tested the effects of NP surface charge on cellular uptake and intracellular trafficking of those NP on eight different cell lines. Intracellular trafficking indicates that some of the positively charged NP could escape from lysosomes after being internalized and exhibited perinuclear localization, whereas the negatively and neutrally charged NP preferred to colocalize in lysosomes (Yue *et al* 2011). These results demonstrate that the NP surface charge also influences their fate inside cells (figure 2). Interestingly, Jing *et al* (2019) showed that when NP have similar charges, the van der Waals forces determine the interaction with the plasma membrane.

NP surface charge does not only influence cell uptake and intracellular trafficking, but it has also been demonstrated that it can influence the cellular phenotype of differentiated cells. A very interesting example was the use of chemically-modified carbon nanotubes (CNT) as a substrate for cultured neurons. It was shown that longer neurites and more elaborate branching were observed on positively-charged carbon nanotubes substrates. The authors highlight that it is possible to control the outgrowth and branching pattern of neuronal processes, by manipulating the charge of the functionalized carbon nanotubes (Hu *et al* 2004). In the case of undifferentiated cells, first attempts to control differentiation through NP addition have been carried out. Li *et al* (2015) functionalized AuNP with an amine ($-NH_2$) and carboxylic acid ($-COOH$) moieties and exposed human bone marrow-derived mesenchymal stem cells (hMSCs) to them. Pristine unfunctionalized AuNP do not inhibit osteogenesis. Moreover, AuNP–COOH reduced alkaline phosphatase (ALP) activity and calcium deposition, but upregulated expression of TGF-β and FGF-2, promoting cell proliferation over osteogenic differentiation in hMSCs. These findings suggest that by understanding in a better way the underlying mechanisms in the nano-bio interface, it is possible to control cell behavior by just modulating NP physical properties, which has a

tremendous technological and biomedical potential (Li *et al* 2015).

3.2. Hydrophobicity

Another parameter to consider is NP hydrophobicity, because it is closely related to cell membrane properties. Coarse-grained molecular dynamic simulations have shown that hydrophobicity enhances the penetration ability of NP into cell membranes and nuclear pores through hydrophobic interactions, while semi-hydrophilic nanoparticles are only found adsorbed in the membrane (Li *et al* 2008). NP hydrophobicity is a property that has been explored to improve current gene therapies. Niikura *et al* (2014) synthesized a bifurcated ligand possessing hydrophobic and hydrophilic arms as a surface ligands for AuNP to deliver small interfering RNAs (siRNA) into HeLa cells. Their results showed that the bifurcated ligand not only promotes cellular uptake, but also enhances AuNP permeation from endosomes into the cytosol, leading to effective gene silencing. This work highlights how changing hydrophobic/hydrophilic forces make it possible to control NP cell internalization to manipulate cell metabolism (Niikura *et al* 2014). Similar hydrophobic effects were observed for poly(L-lactide) functionalized NP in HeLa cells, indicating a great potential to develop new cancer therapies (Samadi Moghaddam *et al* 2015). In addition, the hydrophobicity of NP surface can be unintentionally affected by non-specific interactions with the NP surface, like the protein corona. This is important because it has been observed that charged and/or hydrophobic NP have fewer cellular interactions once they are fouled with proteins. Once fouled, NP may associate with cells through unpredictable interactions.

3.3. Protein corona

Protein corona is a critical and vast issue that has gained interest in recent research. Many excellent reviews about it have been recently published (Capjak *et al* 2017, Jain *et al* 2017, Charbgoo *et al* 2018). Therefore, we will only mention a couple of examples to explain the relevance of the protein corona effect for designing nanomedical devices. Protein corona is a layer formed on the NP surface upon exposure to high protein concentrations, as in biological fluids. Walkey *et al* (2014) have identified protein corona fingerprints formed around a library of 105 types of surface-modified AuNP. Their results suggest that hyaluronan receptors are the primary mediators of nanoparticle–cell interactions. An example of the impact of this is the development of novel medical nanodevices that act like nanocarriers for subcellular- and organelle-level targeting, which are referred to as the third generation of nanomedicines (Yameen *et al* 2014). Kou *et al* (2013) have reviewed some strategies based on nanotechnology for specific organelle targeting. The authors remarked that the main strategy for NP cell targeting is biochemical ligand-coating (Kou *et al* 2013). However, there is evidence that shows that unpredicted NP–protein corona interactions can induce conformational changes on the coated ligands on the NP surface, leading to the exposure of new, unexpected epitopes. This interaction affects NP properties, including cell targeting (Nguyen and Lee, 2017). Moreover, as we previously mentioned, protein corona influences NP size and shape, but it has also been recently demonstrated that size per se influences the NP protein corona due to curvature effects (Lundqvist *et al* 2017). This finding emphasizes the interaction between NP size and the protein corona. The interaction between NP size and the protein corona is poorly understood, even when it has a significant impact on the performance of nanodevices and the consequent cellular response.

3.4. NP coating

NP have been coated with different molecules, depending on the purpose for which they were designed. For example, to enhance their biocompatibility, NP have been coated with biopolymers, such as chitosan and hyaluronic acid, or with synthetic polymers, such as poly(vinyl alcohol), poly(lactic-co-glycolic) acid, PEG, among others. For molecule delivery, NP have been coated with dyes, liposome based-nanoparticles, or PEG, which facilitates the entrance to cells. To avoid immune responses, NP have been coated with PEG or polyacrylic acid. For diverse medical applications, NP have also been coated with quantum dots due to their fluorescence properties, contrasting agents, and antibacterial compounds. Moreover, for gene therapy, NP have been coated both with DNA and RNA. For cell targeting, NP have been coated with a wide variety of biomolecules such as specific ligands and proteins, including antibodies and enzymes. Recently Scaffaro *et al* (2018) published an excellent review of this topic (Scaffaro *et al* 2018). A summary of NP coating strategies is presented in figure 3.

The most common strategy for cell targeting is to attach ligands to the NP surface, such as monoclonal antibodies or peptides. This strategy has been proposed for cancer nanotherapies (Nobs *et al* 2004, Peiris *et al* 2018). The main parameter to consider in the early NP coating design is, of course, the use of the appropriate ligand for a specific receptor. Nevertheless, it is important to consider ligand number, density, and length. There are many efforts in the literature to design NP with the optimal number and length of ligand coating, as we discussed previously based on the work of Decuzzi and Ferrari (2007). In this context, according to Yuan and Zhang (2010), there exists an optimal combination of size and ligand density at which the endocytic times are minimized, and some mathematical models have been proposed to estimate the optimal number of ligands to be attached to a NP (Yuan and Zhang 2010; Zhang *et al* 2015). A recent study of statistical dynamics showed that cellular internalization of NP strongly depends on ligand distribution, and that the cellular uptake efficiency of NP was higher when ligand distribution was uniform. These results also indicate that the optimal ligand distribution associated with the highest cellular uptake efficiency depends slightly on the distribution pattern of ligands and density of receptors, and that the optimal uniform distribution is obtained when receptor

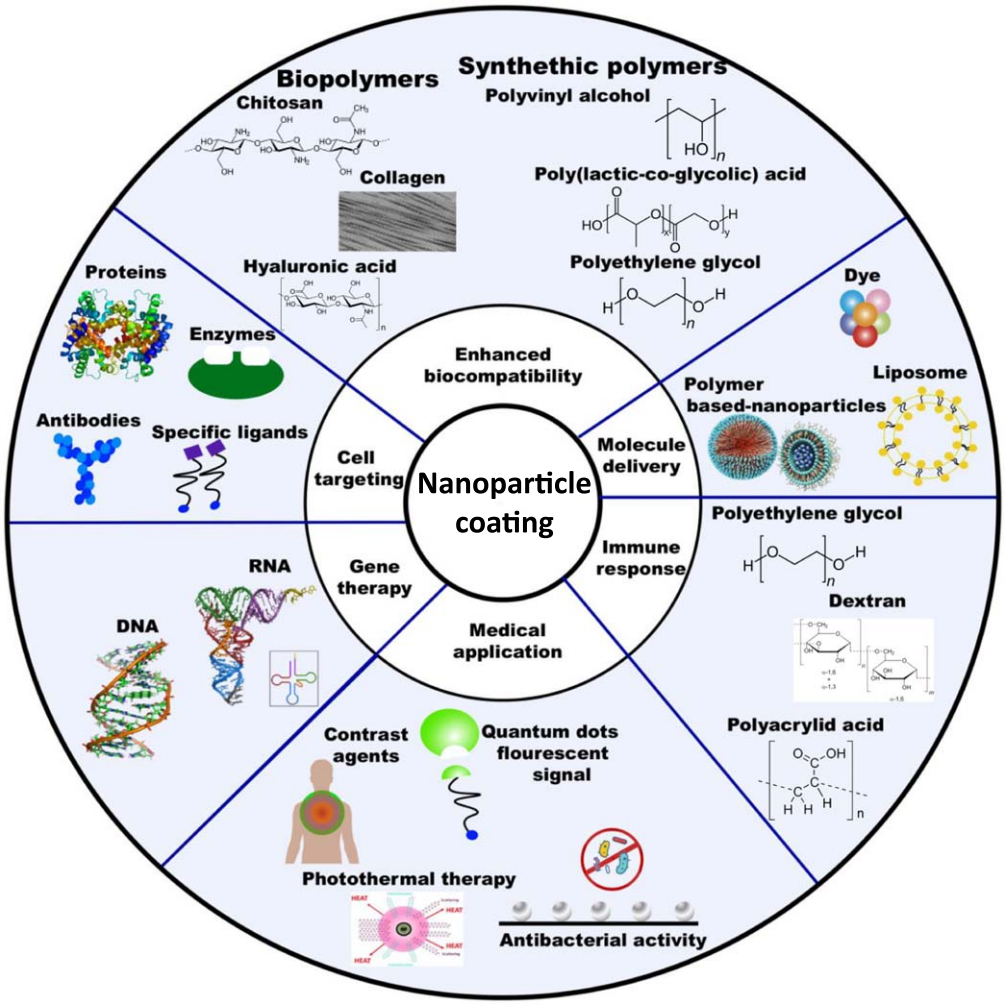

Figure 3. Common NP coating strategies for specific medical purposes.

density is sufficiently high, although this study is limited because the cylindrical shape of NP and the membrane surface tension were not considered. This work proposes that an efficient NP coating strategy is similar to the homogeneous ligand distribution of enveloped viruses (Li *et al* 2017). It is worth highlighting that none of the mathematical models currently available considers NP size, shape (anisotropy), NP dynamics, and ligand distribution simultaneously, needed to provide a guide for NP ligand-coating for a more efficient cell targeting. This lack of knowledge has encouraged many groups to study this problem.

4. Shape

The shape is another important factor that determines the structural, physical, and chemical properties of NP. Different NP shapes affect the electronic, optical, and magnetic properties. Recently, nanotechnology has skyrocketed developing innovative methodologies to produce an extensive catalog of different NP shapes (Zhang *et al* 2018a), such as a lotus leaf-like structure (Hao *et al* 2017), a flower-like structure with wrinkled edges (Kang *et al* 2016), scroll-like cylindrical NP (Avivi *et al* 1999), ellipsoids (Ulrich *et al* 2016), rod-shaped (Ng *et al* 2012), octahedra, 2D-triangles, dumbbells, belts, hexagons (Zhang *et al* 2007), etc. Simple tuning of solvent composition or synthesis conditions can result in alterations of NP shape, offering possibilities for designing a wide variety of NP shapes for diverse applications. These technological advances on NP synthesis can undoubtedly enlarge the spectrum of solutions to improve the function and targeting of nanodevices, but also to provide tools for understanding NP–cell interactions and to elicit the desired cellular responses by manipulating NP physical properties. Decuzzi *et al* (2008) raised the question if NP geometry is relevant for systemic drug delivery. If so, is geometry equally relevant at different length scales (vascular, cellular, and subcellular levels)? Nowadays, there is theoretical and experimental evidence that shows that the shape of NP can be manipulated for specific nanomedical devices, affecting particle margination and interaction with various cell types (Jurney *et al* 2017). For example, a recent exciting study showed that gold nanoflowers possess the most promising non-cytotoxic behavior for mammalian cell cultures, with a high shape-dependent antibacterial activity against *Staphylococcus aureus,* in contrast with their spherical counterpart (figure 4) (Penders *et al*

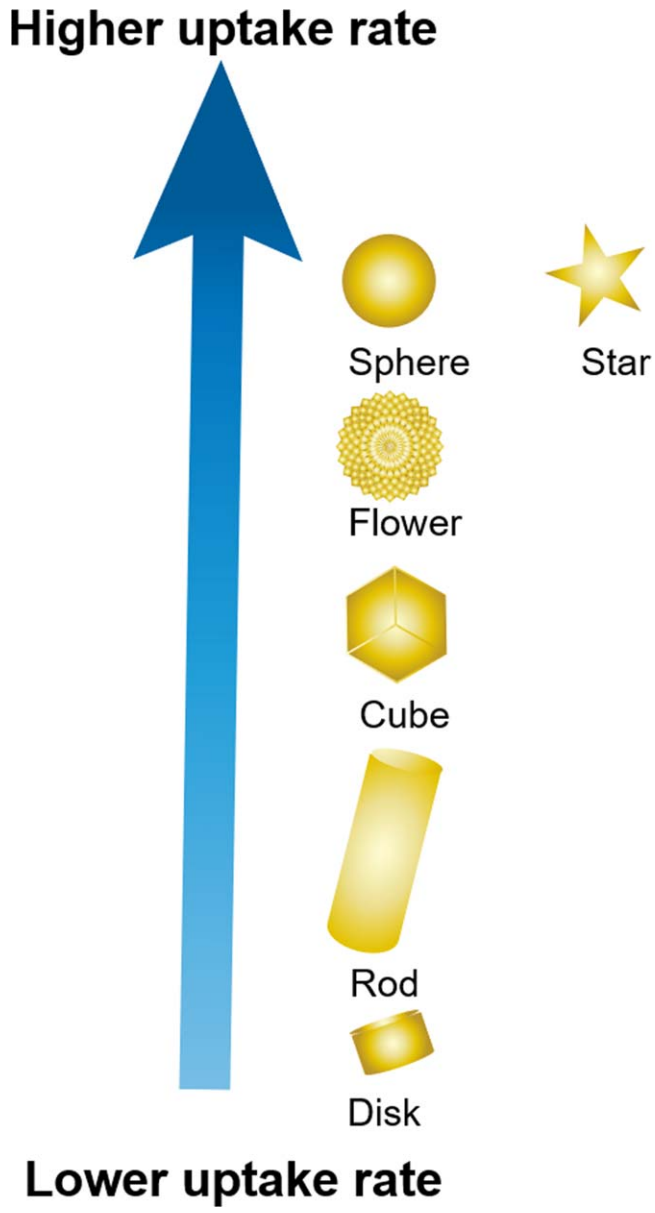

Figure 4. Shape-dependent NP uptake. NP with diverse shapes exhibit different uptake rates, probably due to the ease of bending of cell membrane around the particles. Gold nanostars emerge as an interesting NP shape for novel medical nanodevices due to their high uptake rate, lower cytotoxicity, and antibacterial activity.

2017). Another study showed that the cytotoxicity of AuNP is also shape-dependent. Au nanospheres and nanorods proved to be more toxic than Au nano-star, flower, and prism gold nanostructures in human embryonic kidney cells HEK293T and HeLa cells exposed to concentrations of 32, 100 and 300 μM for 72 h (Woźniak *et al* 2017).

To take advantage of this knowledge for the design of better medical nanodevices, it is crucial to understand how NP geometry impacts cellular internalization, intracellular trafficking, cell cytotoxicity, etc.

4.1. Shape-dependent cytotoxicity

Recent experimental evidence has shown that NP shape also influences their toxicity. Oh *et al* (2010) exposed human lung fibroblasts (IMR90) and mouse alveolar macrophages (J774A.1) to NP of the conductive polymer poly (3,4-ethylene dioxythiophene) (PEDOT) of different shapes. The authors found that the shape and concentration of the PEDOT NP determine the onset of cellular oxidative stress. Moreover, proinflammatory cytokines (interleukin-1 and interleukin-6) and tumor necrosis factor α from macrophages are induced by PEDOT NP in treated cells (Oh *et al* 2010). These findings were recently confirmed by Zhang *et al* (2017) using poly (lactic-co-glycolic acid)-PEG NP (PLGA-PEG NP), which is an FDA approved material for biomedical applications. They exposed the human liver cancer cell line HepG2 to spherical and needle-shaped PLGA-PAG NP. Needle-shaped NP induced significant cytotoxicity measured by the MTT assay, LDH release, and caspase 3 activity as an apoptosis marker. Their study evidenced that the cytotoxicity of needle-shaped NP was induced through the lysosome enlargement. Lysosome disruption activated the signaling pathways of caspase 3 for cell apoptosis, and eventually caused DNA fragmentation and apoptotic cell death. Interestingly, in contrast to spherical-shaped PLGA-PEG NP, no cytotoxicity was detected (Zhang *et al* 2017). Taken together, these findings suggest that cytotoxicity and apoptosis increase with the decreasing NP aspect ratio. However, it should be considered that sharp edges of anisotropic structures can be responsible for the injury of blood vessels (Vácha *et al* 2011). Additional research is needed to elucidate the biophysical mechanism by which NP shape influences cell metabolism. These findings emphasize that shape is an important parameter to consider for efficient and safe medical nanodevices. Depending on the NP application desired, we can choose a specific NP shape. For example, spherical-, star- and flower-shaped AuNP are highly efficient in internalization experiments and can be applied for designing delivery nanosystems. Steckiewicz *et al* (2019) compared the cytotoxicity of gold NP rods (39 $\times$ 18 nm), stars (215 nm), and spheres (6 nm). They found that spheres were the least cytotoxic and stars the most. However, it should be considered that the studied NP had a wide range of sizes.

4.2. Shape-dependent internalization

It is not trivial to relate NP shape to a specific cell internalization mechanism. It has been proposed that NP internalization is a complex manifestation of three shape- and size-dependent parameters: (a) particle surface-to-cell membrane contact area, i.e. particle–cell adhesion, (b) strain energy for membrane deformation, and (c) sedimentation or local particle concentration at the cell membrane particle–cell adhesion (Agarwal *et al* 2013). These parameters influence the probability (limited for the shear stress and the optimal NP size) of the NP to be internalized. Decuzzi *et al* 2008 showed that NP with extremely low or high aspect ratio are not endocytosed, and that the aspect ratio should be within the range suitable

for complete wrapping of particles by the cell membrane (Decuzzi *et al* 2008). In this context, it has been demonstrated that oblate shapes adhere more effectively to the biological substrate than classical spherical particles of the same volume, which can improve the therapeutic efficacy (Decuzzi and Ferrari, 2006). In another study, it was shown by molecular dynamic analysis that spherocylindrical NP are more efficiently endocytosed than spherical-shaped NP. Cells were unable to uptake cylindrical particles due to their sharp ends. It was suggested that NP shape-dependent endocytosis depends on the different surface adhesion energy of each shape (Vácha *et al* 2011). These results are supported by the observations of Agarwal *et al* (2013), who showed that disk-like negatively charged NP of high aspect ratios have higher cellular uptake than nanorods and lower aspect-ratio nanodisks in mammalian epithelial and immune cells (Agarwal *et al* 2013). It was concluded that less strain energy is needed for bending of cell membranes around nanodisks than around nanorods, with a consequent higher cellular uptake. In a later work, Li *et al* (2015a) observed that cellular uptake of nanoparticles depends on their shape, with a higher uptake for sphere > cube > rod > disk, probably due to the ease of bending of the cell membrane around the particles. Authors also found that star-shaped NP can be quickly wrapped by the cell membrane, similar to their spherical counterparts, resulting in a high uptake (figure 4) (Li *et al* 2015a).

Dasgupta *et al* investigated the role of nanoparticle shape and size, as well as membrane bending rigidity and tension on membrane wrapping and cellular uptake. According to their results, rod-like NP were observed in stable endocytic states with small and high wrapping fraction. Interestingly, for high aspect ratios and round tips, the particles enter side-first with their long edge parallel to the membrane. In contrast, for small aspect ratios and flat tips, NP enter tip-first. This work highlights the relevance of NP orientation for interacting with cells (Dasgupta *et al* 2014). In this context, it has been demonstrated that the shape-dependent uptake of NP differs from dynamic uptake experiments. Jurney *et al* (2017) exposed endothelial cells to negatively charged, non-spherical PEG hydrogel particles. Cells were cultured in a microchannel system with a physiologically relevant shear flow rate and were compared with a static cultures. Their results show that larger rod- and disk-shaped NP had a higher uptake compared with the smaller ones, in contrast with the size effect observed for spherical NP in a flow. Moreover, the authors showed that the NP uptake varies on the dynamic and the static culture system (Jurney *et al* 2017). Microfluidic simulations have shown that non-spherical NP have more complex motions, with tumbling and rolling even in typical capillary hydrodynamic conditions. For non-spherical NP in the absence of gravity, a combined effect of three factors has been observed: particle non-spherical shape, its inertia, and particle-wall hydrodynamic interactions. In these conditions, lateral drifting velocity is directly related to the aspect ratio, with a maximum between the two extremes: sphere and disk with aspect ratios of 1 and infinity, respectively (Gavze 1998). This means that increasing rotational and tumbling motions of larger-size non-spherical NP in the flow, play a dominant role in NP margination and cell interaction, compared to Brownian motion, gravity, and cell membrane deformation energy. Moreover, according to the computational simulations of Li *et al* (2012), NP rotation is one of the most important mechanisms that regulate the competition between ligand–receptor binding and membrane deformation. Due to the strong ligand–receptor binding energy, the NP membrane invagination is featured by the rotation of NP to maximize their contact area with the cell membrane. Thus, rotation is one of the most important mechanisms that determines that the endocytosis of NP has shape anisotropy. The kinetics of wrapping are mainly dominated by the orientation of the NP that interacts with the membrane, i.e. the part of the NP with the largest local mean curvature at which the membrane is most strongly bent. This study also demonstrated that the shape anisotropy of NP generates a heterogeneous membrane curvature distribution that induces an asymmetric endocytosis (Li *et al* 2012). In phagocytosis, NP orientation is also important, according to Champion and Mitragotri. Local particle shape, measured by tangent angles, at the point of initial contact dictates whether macrophages initiate phagocytosis or simply spread on particles (Champion and Mitragotri 2006). Taken together, these results can explain the different uptake observed in static versus dynamic cultures exposed to NP with different shapes. Also, these findings invite researchers to be cautious when extrapolating their results of NP shape-dependent uptake in static cultures to dynamic *in vivo* models.

4.3. Shape-dependent intracellular trafficking

It has been suggested in some theoretical works that NP shape is a critical factor in determining the translocation of NP across a lipid bilayer (Yang and Ma, 2010, Ding *et al* 2012), but only few articles relate NP shape with the intracellular trafficking. In a systematic study, Chu *et al* (2015) demonstrated the role of morphological processes in determining the cellular translocation dynamics but also the fate of the NP. Authors found that NP with sharp shapes, regardless of their surface chemistry, size, or composition, can pierce the membranes of endosomes that carry them into the cells, and escape to the cytoplasm, which in turn significantly reduced the cellular excretion rate of NP (Chu *et al* 2015). In this context, Muro *et al* (2008) showed the importance of understanding the mechanisms underlying NP intracellular trafficking. They designed polystyrene nano and microparticles for specific therapeutic needs. The authors observed that spherical polystyrene NP in the nanometer range (100 nm) targeted to enter the cell via adhesion molecule-mediated endocytosis showed a more efficient transport to lysosomes than spherical or elliptical disk polystyrene particles at the micro-size range, which remained for more time in prelylososomal compartments. Taking advantage of these observations, authors functionalized micron-size particles with catalase, an enzyme that converts hydrogen peroxide into water and oxygen. The particles were endocyted and resided for a prolonged time in pre-lysosomal compartments, where they exerted their activity and protected the cell from

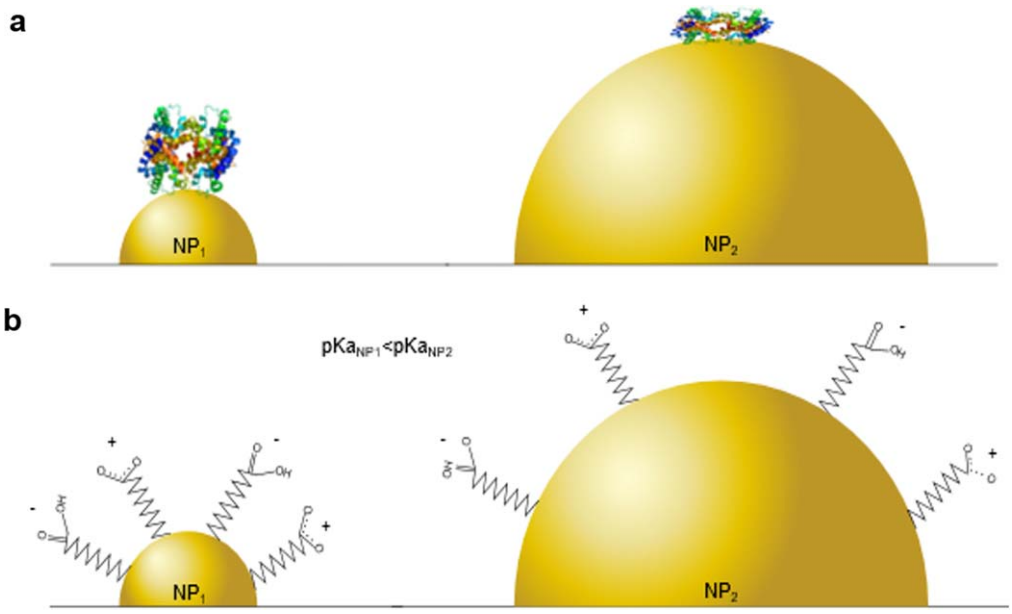

Figure 5. Influence of NP curvature in their coating. NP curvature determines (A) the conformational changes induced in bound proteins and (B) the apparent acid dissociation constants (pKa).

oxidative damage. Meanwhile, nano-sized polystyrene particles were functionalized with acid sphingomyelinase and efficiently delivered to lysosomes as an enzyme replacement therapy for attenuation of accumulated sphingomyelin, a lysosomal storage disorder. This work highlighted that just by varying the size of particles, different subcellular destinations can be achieved (Muro *et al* 2008).

4.4. Interpretation of shape-dependent cellular responses: curvature makes the difference

Anisotropic NP have very complex morphologies with regions of high and low curvatures. It has been shown that temperature, ligand coating, the presence of specific ions, and even protein corona formation can provoke a change in NP shape toward more stable structures (Yoshida *et al* 2011, Satzer *et al* 2015, Fang *et al* 2017, Jana *et al* 2017). This fact remains poorly understood, and it has also been widely neglected in the literature. The geometrical change resulting from a simple external stimulus can have broad implications for the design of medical nanodevices, but also ifor the nterpretation of shape-related cellular responses.

NP curvature modifies the NP coating (figure 5). NP curvature influences the conformational changes induced in protein binding on NP surface (figure 5(a)). Lundqvist *et al* showed that differences in silica NP curvatures (6, 9, and 15 nm of diameters) strongly perturbed the secondary structure of an attached human carbonic anhydrase. NP with a longer diameter allow the formation of larger particle−protein interaction surfaces and cause more significant perturbations of the protein's secondary structure upon interaction (Lundqvist *et al* 2004). In addition, it has been shown that NP local curvature elicits a different ionization state of an adsorbed molecular layer in two opposite NP curvatures, resulting in different charges on the NP surface, especially when acidic ligands are used. The heterogeneity of ligand density immobilized on anisotropic NP surface also influences their dissociation. This means that the apparent acid dissociation constant (pKa) of two NP with the same coating but different curvatures can be different (figure 5(b)) (Wang *et al* 2011).

Many studies have evidenced the heterogeneity of ligand coating density on non-spherical NP. Studies of the spatially dependent kinetics of protein corona formation around Ag nanocubes showed that there are significant differences in protein adsorption at the edges, compared with corners at short incubation times (Miclăuş *et al* 2014). The knowledge about NP coating preference for a specific curvature opens the possibility for generating charge patterns that guide the attachment of NP, proteins or other biomolecules, or even for designing NP self-assembled clusters, as Walker and co-workers demonstrated (Walker *et al* 2013).

The shape-dependent influence of NP reactivity can be easily misattributed to a specific cellular response. However, the real causal relation is possibly the different surface reactivity, rather than any geometrical property (Kinnear *et al* 2017). It is challenging to separate a cell response from the specific physical consequences of NP anisotropy. In this context, theoretical approaches could significantly improve our understanding of the influence of NP shape on membrane interaction, and ligand coating alterations, but due to the complex interactions between size, shape, coating, protein corona, flows, charges, etc, theoretical approaches should be supported by strong experimental evidence to associate more accurately a specific cell behavior.

5. Additional considerations

5.1. Rigidity

Sun *et al* (2015) demonstrated that stiffness is also an essential parameter for designing NP. According to their results, an increment of the NP rigidity, while keeping the same NP chemical composition, size, and surface properties, a higher uptake is favored due to the easier membrane deformation. In contrast, softer NP are trapped in the membrane (Sun *et al* 2015). These works show for the very first time that tuning the rigidity of NP is an appealing way to improve therapeutic efficiency, especially for applications like drug delivery. Otherwise, it has been observed that ovarian cancer cells' nuclear rigidity increases due to the presence of AuNP. These exciting results are the research focus of several groups, as the nuclear rigidity of the cell largely decreases cell migration and could potentially inhibit cancer cell invasion (Ali *et al* 2017). Similar results were obtained in mesenchymal stem cells cultured in the presence of silica NP. This suggests that the effects of silica-based NP may result in the structural reorganization of the cortical cytoskeleton with subsequent stiffness increase and concomitant F-actin content decrease (Ogneva *et al* 2014). Recently, an excellent review was published by Septiadi *et al* (2018) about the interaction between NP and cells from the point of view of bionanomechanics, i.e. the ability of intracellular and extracellular NP to impair cell adhesion, cytoskeletal organization, stiffness, and migration are discussed (Septiadi *et al* 2018).

5.2. Nanopattering

Although plenty of evidence has been gathered, the exact mechanism of topography-induced cellular behavior has not been fully elucidated. Stem cell differentiation modulated by biophysical cues present on nanomaterials, such as nanopatterning and stiffness, has become a fast-growing field with significant implications in regenerative medicine. Cells are capable of sensing nanoscale topographical features and the elasticity of the extracellular matrix that surrounds them. These physical cues are transduced via mechanical forces to signaling pathways that ultimately lead to cell differentiation. Teo *et al* used hMSCs and polydimethylsiloxane (PDMS) nano grafts. It was found that cells growing on patterned PDMS of 250 nm width differentiated into a neuronal lineage, a phenomenon not seen when growing over unpatterned PDMS. Moreover, it was observed that nano grafting width could regulate focal adhesion (FA) spatial organization, leading to changes in the actin cytoskeleton, and causing differential gene expression. FA are multicomponent protein complexes that bridge the cytoskeleton network to the extracellular matrix, it was demonstrated that focal adhesion kinase (FAK) had a crucial role as a signaling molecule to transduce topography signals to the nucleus triggering a series of downstream pathways for neuronal differentiation. It was also proved that although nanopatterning can induce cell differentiation by itself, a synergistic effect is seen when combined with biochemical cues (Teo *et al* 2013). Moreover, Chen and Hsiue (2013) also have demonstrated that carboxylated MWCNTs can induce and maintain neural differentiation of hMSCs without any exogenous differentiating factors, as evidenced by the protein expression. According to the authors' proposal, MWCNTs can promote hMSCs neural differentiation, including up-regulating the neural growth factors; and trapping these neural growth factors to create a suitable environment for long-term neural differentiation (Chen and Hsiue, 2013). Moreover, Kim *et al* (2015) prepared a series of micropatterned geometries of nanosized graphene oxide (NGO) to guide stem cell differentiation. They found that human adipose-derived mesenchymal stem cells (hADMSC) growing in the presence of an osteogenic medium in a linear patterning promoted osteogenesis with high levels of calcification, an indicator of bone regeneration. They observed that biochemical signals, patterning, and the physicochemical properties of NGO synergistically promoted osteoblast differentiation. Meanwhile, a grid NGO pattern facilitated neurogenesis with the highest conversion efficiency reported so far, 30%, a good value since it has been difficult to differentiate hADMSCs into ectodermal neuronal cells. This enhanced neuronal differentiation was attributed to the grid-like pattern that mimics the elongated and interconnected neuronal network (Kim *et al* 2015).

5.3. Cell cycle phase

Another interesting parameter to be considered for a more accurate interpretation of data is the cell cycle phase at which nanoparticle internalization occurs. Kim *et al* (2012) showed that the accumulation of carboxyl-functionalized polystyrene nanoparticles (PS-COOH, 40 nm of diameter) with an overall negative ζ-potential in human lung carcinoma cells (A549) is dependent on their cell cycle phase. While cells in different phases of the cell cycle internalized NP at similar rates, the intracellular NP concentration after 24 h of incubation depended on the cellular cell cycle phase in this order: $G2/M > S > G0/G1$. They also observed that nanoparticles were distributed among daughter cells upon division (Kim *et al* 2012). Similar results are reported by Rees *et al* (2019), who attribute the observed differences on the number of endosomes per cell.

Many NP types have been reported to have the capacity to arrest cells in a specific cell cycle phase. Some of them have been reviewed by Mahmoudi *et al* (2011). In that review, the authors concluded that the various effects on the cell cycle might depend on the intracellular location of the NP (Mahmoudi *et al* 2011). In a later work, Patel *et al* (2016) exposed the human epidermal carcinoma cell line A431 to ZnO NP. Their data demonstrated that ZnO NP did not induce cell cycle arrest in S or $G2/M$ phases. Moreover, they observed the cell cycle-dependent cellular uptake of ZnO NP. The higher uptake was observed in the $G2/M$ phase, compared with other phases (Patel *et al* 2016). This work is one of the few pieces of evidence that shows how the cell cycle phase influences NP uptake. Further research is needed to elucidate how cell cycle phase determines NP uptake, trafficking, and metabolism, and ultimately safety.

6. Conclusions

The overview presented here of the interaction of nanoparticles and nanomaterials with cells is required for the design of medical nanodevices. The considerations presented in this document can also be relevant for the design of macroscale medical devices that can produce NP during their use. Based on the information presented, decisions on how such nanodevices are to be designed to achieve the desired biological function should be made considering the complete biological–nanomaterial-device interaction. The key messages of this review are summarized below. There is not an 'adequate' size for optimal NP internalization, although the best size that most of the literature has proposed is approximately 50 nm. NP size influences the endocytic pathway followed for internalization into cells. Most of the experimental data show that ≈100 nm-sized NP are preferably internalized via clathrin-mediated endocytosis. Otherwise, the rest of the endocytosis pathways were highly dependent on cell and material type. NP with diverse shapes exhibit different uptake rates as follows: spherical-, star- and flower-shaped AuNP > cube > rod > disk, probably due to ease of bending of cell membrane around the particles. This knowledge can be useful for designing cellular delivery systems. Gold nanostars have emerged as an attractive NP shape for novel medical nanodevices due to their higher uptake rate, lower cytotoxicity to mammalian cells, and antibacterial activity. Shape-dependent NP reactivity can be easily misattributed to shape-dependent cellular responses, when the real cause can be the different NP surface reactivity due to the local NP curvature with which they interact with cells. Shape-dependent endocytosis of NP depends on the different surface adhesion energy of each shape but also on the orientation with which they interact with the cell.

Anisotropic NP have different surface reactivity (including heterogeneous ligand coating), which can lead to misattributions about shape-dependent cell responses. NP internalization is a complex manifestation of three shape- and size-dependent parameters: (a) particle surface-to-cell membrane contact area, (b) strain energy for membrane deformation, and (c) sedimentation or local particle concentration at the cell membrane particle–cell adhesion. In dynamic experiments with NP, rotational and tumbling motions of larger-size non-spherical NP in the flow play a dominant role in NP margination and cell interaction, compared to Brownian motion, gravity, and cell membrane deformation energy. The protein corona formed on NP at contact with serum significantly enlarges the NP hydrodynamic radius and changes the NP shape. This can hide important evidence about how NP size influences cellular responses or leads to imprecise conclusions. Positively charged NP have resulted in higher uptake rates and efficiency in diverse cell types. NP charge and hydrophobic/hydrophilic properties might influence cell trafficking and NP fate inside cells. Cytotoxicity and apoptosis increase with the decreasing NP aspect ratio. It has been proposed that an efficient NP coating strategy could be inspired in the homogeneous ligand distribution that enveloped viruses use to achieve infection. The intricate relationship between NP size, shape and protein corona, and how to influence one with the other, especially in the presence of ligands on the NP surface, remains poorly understood. Rotation is one of the most important mechanisms for anisotropic NP endocytosis and phagocytosis. Shape anisotropy of NP generates a heterogeneous membrane curvature distribution that induces asymmetric endocytosis. Increasing the NP rigidity, a higher uptake is favored due to the easier membrane deformation. The cell cycle phase is another important parameter that influences cell response to NP, such as NP uptake rate. However, NP exposure can affect the cell cycle, for example, arresting cells in a specific cycle phase.

7. Future perspectives

Understanding nano–bio interactions is non-trivial, as the physics of nanomaterials is between the frontiers of classical and quantic physics, in the 'mesoscopic scale'. Unlike quantic and macroscopic physics, in the mesoscopic scale, the average behaviors exist but are profoundly affected by fluctuations that have a deterministic origin. Currently, a powerful pool of scientific theories, technologies, and techniques have been developed, and allow us to synthesize a wide variety of nanomaterials *à la carte* with unforeseen applications. There is no doubt that these advances can provide a wide spectrum of solutions to increase and improve the application of nanodevices, and they add extra degrees of freedom to the current understanding of NP-cell interactions and to elicit desired cell responses by manipulating NP physical properties. How far are we from having a sufficient understanding of cell responses mediated by NP interactions for a rational development of safe and effective biomedical nanodevices? First, we must emphasize that there is a lack of systematic reports studying the influence of NP properties other than size and dose. Second, further research is needed to elucidate the influence of NP on the complete cell response, i.e. the synergy between shape, local curvature, cell cycle phase, protein corona, orientation, rigidity, surface coating, etc, to avoid misattributing a cell behavior to an incorrect NP property. In this context, theoretical approaches can be beneficial. For more accurate results, possible synergic effects should be considered and supported by experimental data for a more accurate understanding.

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