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Concepts of nanoparticle cellular uptake, intracellular trafficking, and kinetics in nanomedicine

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ABSTRACT

Nanoparticle-based therapeutics and diagnostics are commonly referred to as nanomedicine and may significantly impact the future of healthcare. However, the clinical translation of these technologies is challenging. One of these challenges is the efficient delivery of nanoparticles to specific cell populations and subcellular targets in the body to elicit desired biological and therapeutic responses. It is critical for researchers to understand the fundamental concepts of how nanoparticles interact with biological systems to predict and control *in vivo* nanoparticle transport for improved clinical benefit. In this overview article, we review and discuss cellular internalization pathways, summarize the field`s understanding of how nanoparticle physicochemical properties affect cellular interactions, and explore and discuss intracellular nanoparticle trafficking and kinetics. Our overview may provide a valuable resource for researchers and may inspire new studies to expand our current understanding of nanotechnology-biology interactions at cellular and subcellular levels with the goal to improve clinical translation of nanomedicines.

Contents

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1. Introduction

The design and medical application of nanoparticles for diagnosis and treatment of diseases represent an important area of current nanotechnology research. This research field has been widely referred to as nanomedicine [1]. In nanomedicine, researchers engineer nanoparticles, for example, as delivery vehicles for therapeutics or imaging agents with the ultimate goal to improve clinical outcomes [2]. To achieve this goal, researchers need to be able to efficiently deliver nanoparticles to diseased sites in the body with cellular specificity and oftentimes subcellular precision [3]. Such efficient and effective nanomedicine delivery requires full control over the nanoparticle transport in the body. However, this level of control has not been achieved yet and is one of the greatest challenges in nanomedicine research [4].

Addressing this challenge is a major quest in the field which emphasizes the need to better understand the fundamental concepts of how nanoparticles interact with biological systems [5]. These nanotechnology-biology (*i.e.*, nano-bio) interactions are complex, dynamic, and multiparametric, which poses substantial obstacles for the engineering of effective nanomedicines [6]. Factors that contribute to this complexity are manifold and include: (i) a nanoparticle's physicochemical properties, including size, shape, surface chemistry, composition, architecture, density, and modulus; (ii) the biological and biochemical environments, including type of organ/tissue, biomolecular milieu and composition, pH, and other biochemical factors; and (iii) the interplay and interactions between these individual nanoparticle properties and biological/biochemical parameters, including the kinetics of nano-bio interactions [7].

While researchers are able to synthesize colloidal nanoparticles in the laboratory with precise physicochemical properties and functions, these deliberately designed nanoparticle characteristics may change substantially upon introduction of nanoparticles into a biological environment [8,9]. This phenomenon can be observed, for example, when nanoparticles are administered into the body through intravenous injection. Upon contact with blood, serum proteins adsorb non-specifically onto the nanoparticle surface to form a so-called protein corona [10,11]. The protein corona alters nanoparticles' physicochemical properties by providing them with an unintentional biological identity [12]. Ultimately, this biological identity determines a nanoparticle's interactions with biological systems, including organs, tissues, cells, and subcellular organelles [13–16]. Therefore, nanoparticle *in vivo* transport and biodistribution are largely controlled by this biological identity rather than the deliberately engineered synthetic nanoparticle characteristics [17,18].

The fact that a nanoparticle's physicochemical properties may change significantly upon biological exposure imposes major challenges for the engineering of nanomedicines. To advance our current understanding and to develop fundamental concepts needed for the design of more effective nanomedicines, researchers have started to describe and decipher essential mechanisms of how nanoparticles interact with biological systems. These studies can be divided into three categories: (i) nanoparticle interactions at organ and tissue levels; (ii) nanoparticle interactions at cellular and subcellular levels; and (iii) nanoparticle interactions with biomolecules and biochemical parameters. We focus in our review article on the second category, *i.e.*, cellular and subcellular interactions of nanoparticles, and refer interested readers to excellent overview articles and original papers that cover nano-bio interactions at organ, tissue, and biomolecular levels [11,19–25].

To maximize clinical benefits of nanomedicines while minimizing side effects, researchers require profound understanding of nanoparticles' cellular and subcellular interactions [19]. An intriguing example is the engineering of nanoparticles that are able to distinguish between healthy and diseased cells through the use of precise biomolecular recognition strategies [26,27]. To achieve this level of cellular identification and discrimination, a nanoparticle surface can be decorated with specific biomolecular ligands that can recognize and bind to complementary cell surface receptors on targeted cells [28]. The idea behind this concept is that upon recognition nanoparticles may deliver their payloads (*e.g.*, active pharmaceutical ingredients; APIs; and imaging agents) preferentially to diseased cells while leaving healthy cells mostly unaffected. As some types of nanoparticle payloads require delivery to specific intracellular targets for maximizing efficacy, it is critical for researchers to understand and explore nanoparticles' cellular interactions, intracellular trafficking pathways, and corresponding kinetics to ensure targeted delivery [29–33].

In this review, we describe the field's understanding of three distinct aspects of nanoparticle-cell interactions: (i) nanoparticle cellular uptake; (ii) nanoparticle intracellular trafficking; and (iii) underlying kinetics of these cellular and subcellular nano-bio interactions. We hope that our review of these important concepts provides a valuable resource to researchers in the nanomedicine field and inspires new research to further enrich our knowledge of cellular and subcellular nanoparticle interactions. With improved knowledge and understanding, better control over nanoparticle transport in the body may be achieved, which could ultimately result in improved clinical benefits of nanomedicines.

2. Cellular uptake of nanoparticles

Cellular uptake of nanoparticles involves highly regulated mechanisms with complex biomolecular interactions to overcome the cell plasma membrane. This biological membrane acts as a barrier and separates a cell's interior from the outside environment. Structural and biomolecular membrane characteristics (*i.e.*, phospholipid-based bilayer membrane littered with proteins and other biomolecules) result in an overall negative charge of the plasma membrane with few cationic domains and selective permeability to ions, (bio)molecules, and nanoparticles. For nanoparticles to achieve cellular entry, they need to overcome the cell plasma membrane. Knowing how nanoparticles enter cells is important, as the underlying uptake pathways determine a nanoparticle's function, intracellular fate, and biological response [34–36].

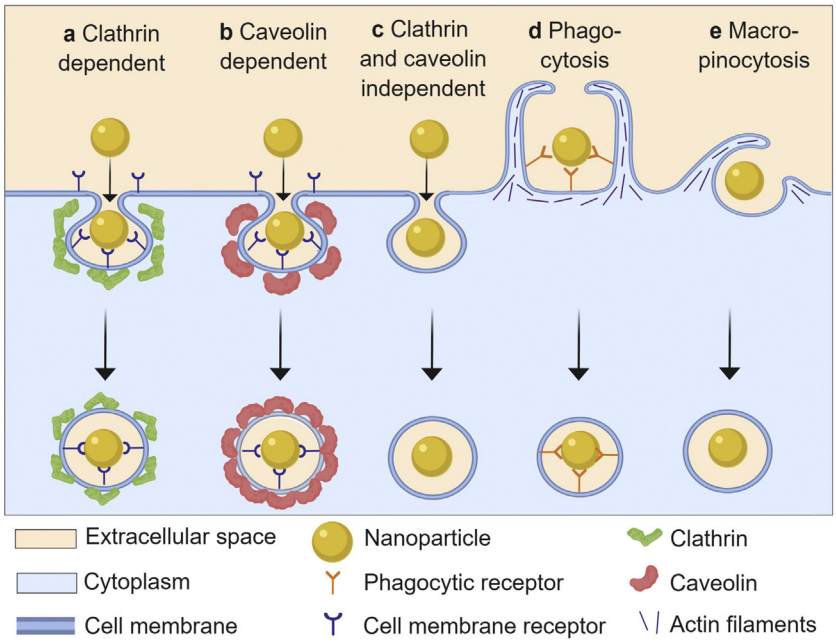

Fig. 1. Schematic overview of nanoparticle uptake pathways *via* endocytosis. Multiple different pathways exist for cellular entry of nanoparticles *via* endocytosis mechanisms: (a) clathrin-dependent; (b) caveolin-dependent; (c) clathrin- and caveolin-independent; (d) phagocytosis; and (e) macropinocytosis pathways. These nanoparticle cell uptake pathways are mechanistically distinct and highly regulated at the biomolecular level. The pathway by which nanoparticles enter cells is important, as it determines intracellular nanoparticle transport and corresponding biological response and therapeutic effect.

Multiple different cellular entry routes are available for nanoparticles to cross a cell's plasma membrane during *in vivo* and *in vitro* cell exposure. These routes can be categorized into two general groups: (i) endocytosis-based uptake pathways (Fig. 1); and (ii) direct cellular entry of nanoparticles (Fig. 2). The field's understanding of these nanoparticle cell entry pathways is currently evolving, as researchers seek to further elucidate fundamental mechanisms of how nanoparticles gain access into cells. In the future, this type of research may enable more efficient and targeted uptake of engineered nanoparticles by desired cells. We will focus this chapter on summarizing the most important cellular uptake pathways and provide examples from recent literature on how nanoparticles enter cells.

2.1. Endocytosis-based pathways

Endocytosis is an umbrella term used to describe multiple different pathways and mechanisms of how nanoparticles can enter cells. These pathways can be differentiated into five mechanistically distinct classes: (a) clathrin-dependent endocytosis; (b) caveolin-dependent endocytosis; (c) clathrin- and caveolin-independent endocytosis; (d) phagocytosis; and (e) macropinocytosis (Fig. 1). From a biomolecular perspective, these uptake pathways are highly regulated and mediated by different types of lipids and transport proteins (*e.g.*, lipid rafts, clathrin, dynamin, caveolin, and pattern recognition receptors). Upon endocytosis, nanoparticles are typically confined within intracellular vesicles, such as endosomes, phagosomes, or macropinosomes, and

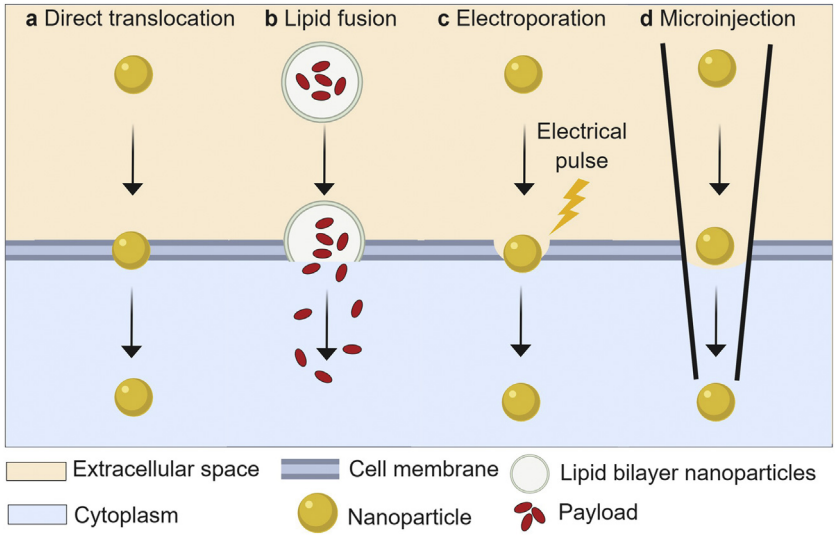

Fig. 2. Schematic overview of nanoparticle cytoplasmic delivery pathways and strategies. Major pathways and strategies for nanoparticles to cross the cell plasma membrane for direct cytoplasmic entry include: (a) direct translocation; (b) lipid fusion; (c) electroporation; and (d) microinjection. Each of these pathways allows nanoparticles to directly enter the cell's cytoplasm. Direct translocation and lipid fusion are dependent upon physicochemical properties of the nanoparticles. Electroporation strategies use electrical pulses to disrupt the cell plasma membrane, while for microinjection strategies the plasma membrane is punctured by a microscopic needle to inject nanoparticles directly into the cytoplasm.

therefore do not have direct and immediate access to the cytoplasm or cellular organelles (Fig. 1). Endosomal vesicles also play critical roles in innate and adaptive immunity as they are important sites for toll-like receptors and major histocompatibility complexes [37,38].

2.1.1. Clathrin-dependent endocytosis

Clathrin-dependent endocytosis is a major pathway for nanoparticle cellular entry and is initiated by the clustering and binding of nanoparticle surface ligands to corresponding cell membrane receptors (Fig. 1a). A wide variety of cell membrane receptors are shared across many cell types (*e.g.*, transferrin receptors, low-density lipoprotein receptors, epidermal growth factor receptors, and β2 adrenergic receptors) and are involved in clathrin-dependent endocytosis [39]. Clathrin-dependent endocytosis is a complex multistep process that includes: (i) nucleation of cytosolic proteins involved in endocytosis to form a coated pit; (ii) plasma membrane bending and invagination; (iii) scission (*i.e.*, cutting and separation of the neck of invagination from the plasma membrane to form an intracellular vesicle); and (iv) uncoating and recovery of the endocytotic proteins from intracellular vesicle [40].

The clathrin-dependent endocytosis pathway results in the entrapment of nanoparticles in intracellular vesicles which exhibit sizes of approximately 100–500 nm [41]. Such vesicles are pinched off the membrane with the help of conformational changes from a GTPase enzyme known as dynamin [42]. Upon scission from the membrane, these vesicles transport typically with the help of intracellular actin filaments to endosomes [43,44]. Endosomes are either recycled or eventually fuse with lysosomes leading to enzymatic breakdown of the engulfed vesicular contents and payloads. Therefore, clathrin-dependent endocytosis provides a pathway for nanoparticles to enter a cell's endolysosomal system. This pathway can be exploited as reported by Benyettou and coworkers. The researchers modified silver nanoparticles to deliver two anticancer therapeutics, doxorubicin and alendronate drugs, to HeLa cancer cells *in vitro* [45]. Upon cellular uptake and lysosomal entrapment, the nanoparticles released their drug payloads in response to the low pH of lysosomes. The anti-cancer activity exhibited by this nano-based drug combination strategy outperformed the efficacy of both drugs when administered individually. We describe other strategies that exploit low pH and enzymatic activity of late stage endosomes/lysosomes to enhance therapeutic responses of intracellular nanoparticles in our intracellular trafficking section.

2.1.2. Caveolin-dependent endocytosis

Caveolin-dependent endocytosis is another important receptor specific nanoparticle internalization pathway that relies on caveolin-coated plasma membrane invaginations termed caveolae (Fig. 1b) [46,47]. Caveolae are flask-shaped vesicles with diameters of 50-100 nm that are stabilized by a caveolin protein based coat [48]. Upon uptake and activation of a complex signaling cascade, caveolin-coated vesicles are transported through the cytoplasm. Typical intracellular destinations of caveolin-based vesicles include the Golgi apparatus and the endoplasmic reticulum [49]. For this reason, caveolin-dependent nanoparticle endocytosis may be a valuable pathway to explore, if researchers seek to achieve intracellular/organelle targeting. Reports have shown that specific nanoparticle surface engineering strategies favor cellular internalization *via* caveolin-dependent endocytosis and typically use nanoparticle surface ligands such as folic acid, albumin, and cholesterol [19]. Recent work by Xin *el al.* exploited caveolin-dependent endocytosis for efficient cytosolic delivery of microRNAs. These nucleic acids were able to bypass lysosomal entrapment to enter a cell's cytosol for downstream inhibitory effects and silencing of KRAS [50].

Caveolin-dependent endocytosis has also been reported to result in transcellular transport of caveolae. This transcellular transport is referred to as transcytosis. Recent studies have focused on exploring caveolin-mediated transcytosis in specific types of cells, including endothelial cells [51–54]. As endothelial cells line the inner surface of blood vessels, these transcytosis-based pathways may allow systemically administered nanoparticles to enter endothelial cells *via* caveolae formation and to cross the endothelium by transcytosis. Such a caveolae-based shuttle mechanism could transport nanoparticles and corresponding payloads actively across the endothelial barrier and may benefit the delivery of therapeutic nanoparticles and their cargoes to diseased tissues in the body for improved efficacy.

2.1.3. Clathrin- and caveolin-independent endocytosis

Virus-like particles and other types of nanoparticles can penetrate the cell plasma membrane and enter cells without relying on clathrin- and caveolin-dependent pathways (Fig. 1c). One suggested route for such clathrin- and caveolin-independent cellular entry involves lipid rafts, which are cholesterol and sphingolipid-rich domains within the plasma membrane that undergo endocytosis when prompted [55]. Lipid raft-mediated endocytosis is a prevalent pathway in immunological scenarios, where lymphocytes internalize and process interleukins [48]. Additionally, specific ligands, such as cholera toxin B and SIV40 bind to lipid rich areas on the cell plasma membrane that undergo lipid raft-mediated endocytosis [56]. Recent studies have pointed towards a lipid raft-mediated endocytosis pathway for the internalization of nanoparticles modified with particular cell-penetrating peptide (CPPs) and nucleic acids [57,58]. It has been suggested that lipid raft, actin cytoskeleton, and cholera toxin subunit B (CTB) mediated endocytosis may be summarized as actin cytoskeleton and cholera toxin subunit B (CTB) pathways [59].

2.1.4. Phagocytosis

Phagocytosis is an uptake process exercised by immune cells, including macrophages, dendritic cells, neutrophils, and B lymphocytes. One of the main roles of phagocytosis is to clear pathogens, diseased cells, and synthetic/biological materials that are foreign to the body [60]. Nanoparticle phagocytosis is typically initiated by physical binding to phagocyte cell surface receptors (Fig. 1d). Examples for these cell surface receptors include: Fc receptors, mannose receptors, scavenger receptors, and complement receptors. Armed with these different types of plasma membrane receptors, phagocytes readily recognize and clear nanoparticles with high efficiency from circulation [61,62]. Recognition and clearance of nanoparticles by phagocytes is mediated by opsonization and adsorption of immunoglobulins, complement proteins and/or other serum proteins onto the nanoparticle surface. Following cellular uptake by phagocytes, nanoparticles are trapped within phagosome vesicles that eventually combine with a lysosome to form a structure known as a phagolysosome. Phagolysosomes are able to enzymatically and biochemically digest foreign "non-self" materials, including nanoparticles [63,64].

Since phagocytosis is a highly efficient clearance mechanism for opsonized nanoparticles, it represents a significant challenge for the engineering of effective nanomedicines. Intravenously administered nanoparticles typically undergo rapid opsonization upon contact with blood [65,66]. These opsonized nanoparticles are then efficiently and rapidly sequestered by macrophages and other phagocytic cells of the mononuclear phagocyte system (MPS) [25]. Up to 99% of a systemically administered nanoparticle bolus dose may be sequestered by the MPS system [65]. Moreover, tissue resident macrophages, such as tumor-associated macrophages, have been shown to uptake cancer cell-targeted nanoparticles to a higher extent than malignant cells [17].

To reduce nanoparticle MPS sequestration, nanoparticle surface modifications have been developed to minimize nanoparticle opsonization [16]. One of these surface engineering strategies uses poly(ethylene glycol) (PEG) to coat nanoparticle surfaces [67]. The PEG surface density and its degree of polymerization may affect nanoparticle opsonization and blood circulation times [68]. A downside of using polymers, such as PEG, is their potential immunogenicity. Repeated administration of PEGylated nanoparticles may result in accelerated nanoparticle blood clearance due to the formation of PEG-specific

antibodies [69,70]. Other recent work has shown that nanoparticles displaying "markers of self" surface ligands (*e.g.*, CD47 peptides) can reduce phagocytotic nanoparticle uptake [71,72]. While these strategies are intriguing approaches to control nanoparticle interactions with phagocytes, there is a need to explore new methods to further minimize immunogenicity and to control unintended phagocytosis of administered nanoparticles.

2.1.5. Macropinocytosis

Macropinocytosis represents a class of non-specific cellular uptake mechanisms that are characterized by engulfment of extracellular fluids and solutes through actin-stabilized plasma membrane extensions (Fig. 1e) [73]. Unlike other endocytotic pathways shown in Fig. 1, macropinocytosis is initiated *via* actin signaling and subsequent membrane ruffling [48,74]. Through this pathway, nanoparticles and other ingested components become trapped within vesicle structures termed macropinosomes. These vesicles may range in size from approximately 0.5 to 1.5 μm [75]. Macropinosomes have been reported to be leaky intracellular vesicles which may allow entrapped nanoparticles to escape before lysosomal degradation [76,77].

Macropinocytosis is an important mechanism required for the proper protective functions of the immune system. For instance, immature dendritic cells constitutively macropinocytose extracellular contents for antigen presentation as part of their sentinel function [78]. By virtue of this behavior, immature dendritic cells are excellent candidates for vaccine targets. Hirosue and coworkers engineered a polymer-based nanoparticle vaccine linked to peptide antigens that indirectly targeted immature dendritic cells [79]. The nanoparticle formulation enhanced antigen-cross presentation and boosted vaccine efficacy by relying on immature dendritic cells with strong macropinocytotic activity. Likewise, macrophages are also known to engage in macropinocytosis. Recently, Nab-paclitaxel (a nanoparticle albumin-bound formulation of the cancer drug paclitaxel) was shown to be engulfed by macrophages *via* macropinocytosis [80]. Importantly, this form of uptake was reported to shift tumor-associated macrophage (TAMs) polarization towards the M1 immunostimulatory phenotype. *In vivo* application of this strategy in mouse tumor models showed an increase in M1 phenotype-like TAMs upon administration of Nab-paclitaxel compared to control groups. Such nanoparticle platforms may have promising potential to diminish cancer's ability to evade immune surveillance by intratumoral immunomodulation.

2.2. Direct cytoplasmic delivery of nanoparticles

Typically, direct access of nanoparticles to the cytoplasm is not observed upon endocytosis-based cellular entry. However, such direct access can be achieved by alternative nanoparticle delivery pathways as shown in Fig. 2. Nanoparticles can cross the cell plasma membrane *via* biochemical or physical means to directly enter the cytoplasm. Nanoparticles that are freely dispersed within the cytoplasm have the opportunity to target and engage subcellular organelles and intracellular structures to elicit deliberate biological responses and medical functions.

2.2.1. Cytoplasmic entry by direct translocation

Following direct translocation pathways, nanoparticles may disrupt the cell plasma membrane by engaging with lipid bilayer molecules to directly transport into the cytoplasm. This route avoids endosomal entrapment and energy-dependent transport mechanisms to gain access to the cell's cytoplasm (Fig. 2a) [81].

Computational models have simulated and elucidated aspects of nanoparticle diffusion through lipid bilayer membranes [82–85]. Based on these studies, nanomedicine researchers have been able to translate *in silico* information and modeling to *in vitro* findings. For example, polymeric nanoparticles with the same surface chemistry and various morphology exhibited different transport paths across the plasma membrane [86]. Both rod and worm-like nanoparticles diffused through the cell plasma membrane more efficiently than spherical micelles.

Using semiconductor quantum dot nanoparticles with a size of approximately 8 nm and zwitterionic surface chemistry, direct translocation of was obsereved in red blood cells [87]. This quantum dot internalization process did not lead to visible pore formation within the cell plasma membrane. Surface-enhanced infrared absorption spectroscopy data suggested that zwitterionic quantum dots entered by means of lipid bilayer softening resulting in subsequent flexible membrane confirmations.

Other research relying on zwitterionic ligands used gold nanoparticles with diameters of 2–4 nm to observe direct diffusion through the plasma membrane of HeLa cells *in vitro*. Interestingly, slightly larger zwitterionic gold nanoparticles with diameters of approximately 6 nm were internalized *via* caveolin/lipid-raft endocytosis, indicating a nanoparticle size dependent effect on cellular internalization [88].

In a different sutdy, Jewell *et al.* sought to understand how the direct entry of monolayer nanoparticles was impacted by cargo size and structure [89]. First, the researchers coated ~5 nm gold nanoparticles with monolayers of a 1:1 mixture of 11-mercapto-1-undecanesulphonate and 1-octanethiol such that different nanoscale arrangements formed "striped" domains on the nanoparticle surface [90]. Next, double stranded and single stranded DNA of varying lengths were chosen as model payloads that were then affixed to the monolayer gold nanoparticles through thiol linkages. After inhibiting endocytosis, the DNA-striped monolayer nanoparticles were internalized by murine melanoma cells. Ultimately, these nanoparticles were seen to deliver various lengths and types of DNA payloads to cells independent of endocytosis due to their unique surface chemistry.

Another important strategy for direct nanoparticle translocation across the cell plasma membrane relies on the use of cell penetrating peptides (CPPs) as nanoparticle surface ligands. These CPPs ligands are short amino acid sequences (typically less than 40 amino acids) that can traverse cell membranes [91]. Much research has been conducted in recent years to elucidate the specific mechanisms for CPP-mediated nanoparticle entry into cells. Our understanding of these mechanisms is still evolving, but studies have suggested multiple different pathways to be involved in cellular uptake, including endocytosis and direct translocation. Factors that seem to regulate the relevance of these pathways include: (i) the type of nanoparticle that CPPs are attached to; and (ii) local concentrations of lipids and peptides in the plasma membrane [92–94]. Frequently used examples of CPPs for biomedical applications are: TAT, penatratin, arginine-rich sequences, TP10, pVEC, and MPG [94–97]. Upon endocytosis of CPP-decorated nanoparticles, endosomal escape may be achieved *via* CPP-mediated vesicle membrane disruption [98,99]. This provides a pathway for endocytosed nanoparticles to enter a cell's cytoplasm. Endosomal escape of endocytosed nanoparticles is a prerequisite for downstream intracellular targeting of subcellular organelles and other compartments, which we discuss in more detail in Chapter 4.

2.2.2. Cytoplasmic entry by lipid fusion

Lipid fusion is the process by which some types of lipid bilayer coated fuse with a cell's plasma membrane (Fig. 2b) [100]. After membrane fusion, the encapsulated contents within the nanoparticle, for example, proteins, nucleotides, and small molecule payloads, are delivered directly to the cytoplasm [101,102]. One group of researchers exploited this pathway for efficient gene knockdown *via* cytoplasmic siRNA delivery [103]. Lipid fusion was accomplished with silicon nanoparticles that were shrouded in a fusogenic liposomal shell. With their construct, Kim *et al.* saw decreased levels of a proinflammatory marker in macrophages (IRF5) which enabled phagocytic clearance of *Staphylococcus aureus* pneumonia and enhanced survival in mice subjected to infection. Recent work probing the lipid-lipid fusion interface has demonstrated that nanoparticles with an amphiphilic organic shell and gold

core exhibit a size-dependent lipid fusion behavior [104]. In the event of endocytosis, Yuba and coworkers delivered immunotherapies to dendritic cells with a liposome antigen delivery system that fused with endosomal membranes for an effective therapy in a murine ovalbumin (OVA)-expressing lymphoma model [105].

2.2.3. Electroporation

Electroporation strategies physically disrupt a cell's plasma membrane upon application of electrical pulses (Fig. 2c). This leads to the formation of transient pores within the membrane through which nanoparticles can transport through from the extracellular space into the cytoplasm. Membrane pore formation generated *via* electroporation can be controlled through fine tuning the electrical pulse (*e.g.*, pulse duration and voltage) such that the newly formed pores do not impact cell viability [106]. Electroporation has been shown to successfully deliver nanoparticles with applications in imaging and genetic engineering. Kim *et al.* used mesoporous silica-coated hollow manganese oxide nanoparticles to label and track adipose derived mesenchymal stem cells [107]. Upon electroporation with an electrical pulse of ~100 V followed by nanoparticle cell entry, the mesenchymal stem cells displayed enhanced contrast in magnetic resonance imaging (MRI) *in vitro* and *in vivo* over the course of 14 days. In a different study, lipid-based nanoparticles efficiently delivered siRNA *via* electroporation (electrical pulse of ~200 V) that silenced PD-L1 and PD-L2 expression on human-monocyte derived dendritic cells [108]. To further demonstrate clinical utility of this method, researchers also reported successful delivery of target antigen mRNA that boosted antigen-specific $CD8^+$ T-cell responses *ex vivo*. Recent studies have shown that electroporation and subsequent transfection can be performed in a high-throughput manner [109,110]. Such technologies may facilitate experiments that require direct cytoplasmic delivery, including gene regulation studies, to help overcome intracellular delivery barriers.

2.2.4. Microinjection

Microinjection strategies are characterized by directly injecting small volumes of nanoparticles into the cytoplasm with the help of specialized microinjectors [111]. With this strategy, cellular and intracellular membrane barriers can be overcome for immediate access of injected nanoparticles to the cytoplasm. As individual cells need to be injected with nanoparticles on a cell-per-cell basis, the throughput of this technique is limited. However, despite being a technically demanding, laborious, and difficult to execute method, microinjection can be a valuable tool to gain nanotoxicology information by excluding extracellular alteration of nanoparticle physicochemical properties (*e.g.,* protein corona formation). This was seen by the microinjection of inorganic nanoparticles into HeLa cancer cells which was used to evaluate the counteractive measures cells develop in the presence of foreign nanomaterials [112].

Microinjection was also used as a tool to investigate ligand density effects on organelle targeting, such as targeting of a cell's nucleus with peptide-modified quantum dots [113]. Additional work employing microinjection uncovered that nanoparticle-mediated gene delivery can be inhibited through lysosomal capturing triggered by autophagy [114].

Our overview of major nanoparticle cell uptake pathways demonstrates that there are many different routes available for nanoparticles to enter cells. As shown exemplarily for gold nanoparticles in Table 1, nanoparticle cellular entry has been linked to multiple different pathways even if the nanoparticle core material (*i.e.*, gold) is kept constant. The relative contribution of these pathways depends on many material design and biological factors, such as surface chemistry, size, shape, and cell type. These data highlight the complexity of nano-bio interactions that are involved in cellular entry of nanoparticles. The relative importance and contribution of individual uptake pathways is not always clear and requires supplemental investigation for many studies. To elicit further control over nanoparticle transport and cellular uptake in biological settings, promoting nanoparticle cellular entry through a singular, defined uptake route is a key quest in nanomedicine research. In Chapter 3, we explore nanoparticle design parameters and how these characteristics affect cellular entry of nanoparticles.

3. Mediating nanoparticle uptake through material design

As shown in Table 1 for gold nanoparticles, cells internalize nanoparticles through multiple different uptake routes even if the nanomaterial is kept constant. These findings suggest that biological factors, including cell type, affect nanoparticle uptake pathways significantly (Table 1).

A study by Saha and coworkers showed that healthy and diseased cells uptake nanoparticles using different pathways. In more detail, healthy mammary epithelium cells and cancerous HeLa cells were each incubated with four different 10-nm cationic monolayer-modified gold nanoparticles [115]. The researchers demonstrated that HeLa cancer cells and healthy epithelium cells employed different mechanisms for nanoparticle internalization despite identical surface modifications of nanoparticles. Altogether, these data suggest that cancer cells may employ various pathways to internalize nanoparticles in contrast to non-malignant cells. This could potentially provide an avenue for improved nanoparticle-based cancer diagnostics and therapies. Additionally, recent reports indicate that female and male cells from various tissues exhibit different nanoparticle internalization patterns [116].

To better study and understand which specific pathway is crucial for nanoparticle uptake by cancer cells, researchers have started to reduce

Table 1
Examples of different cellular uptake pathways of gold nanoparticles in tissue culture for both cancerous and non-cancerous cells.

Major uptake pathways	Cell line	Nanoparticle surface modification	Gold nanoparticle core size [nm]	Ref.
CVE	HeLa	Cysteine-cyan5	4.5	[304]
CVE	HeLa	Cationic monolayer	2	[305]
CVE and lipid rafts	C166	Nucleic acids	10	[251]
CVE, macropinocytosis	A549	Poly(isobutylene-alt-maleic anhydride)	13	[306]
CDE	MRC-5	Coating with fetal bovine serum (FBS)	20	[307]
CDE, CCIE	HUVEC	Citrate	80	[308]
CDE	MCF10	Cationic monolayer	2	[305]
CCIE	HeLa	Cationic monolayer	2	[305]
Phagocytosis	Murine peritoneal-isolated macrophages	5-aminovaleric acid, L-DOPA, Melatonin, Serotonin-HCl,	30-50	[309]
Direct translocation	Mouse dendritic cells	MUS/OT with "striped" domains	4-5	[310]
Direct translocation	HCT-116	Glutathione/glucose	5	[311]
Electroporation	NIH/3T3, K562	Polyethyleneimine, DNA plasmids, siRNA	5-40	[312]
Microinjection	Murine 2-cell embryos	"Ligand free"; no deliberate surface modification	11	[313]

Abbreviations: CDE: Clathrin-dependent endocytosis, CVE: Caveolin-dependent endocytosis, CCIE: Clathrin/caveolin-independent endocytosis, HeLa: human cervical cancer cells, C166: mouse endothelial cells, A549: adenocarcinoma human alveolar basal epithelial cells, MRC-5: human lung fibroblasts, HUVEC: human umbilical vein vascular endothelium cells, HCT-116: human colorectal carcinoma, NIH/3T3: mouse embryo fibroblasts, K562 human chronic myelogenous leukemia, L-DOPA: (S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid, Melatonin: N-acetyl-5-methoxytryptamine, Serotonin HCl: 5-hydroxytryptamine hydrochloride, MUS/OT: 11-mercapto-1-undecanesulphonate and 1-octanethiol, siRNA: small interfering Ribonucleic Acids

the activity of key proteins involved in endocytosis *via* siRNAs [117]. For example, cancerous HeLa cells with reduced expression of the caveolin-1 protein experienced a ~30% reduction in PEGylated nanoparticle uptake. Meanwhile knocking down CDC42 (a key protein for macropinocytosis) reduced the uptake of PEGylated nanoparticles almost by half [117].

Considering such complex differences in cellular uptake among various types of cells, nanomedicine researchers focus on manipulating nanoparticles to exhibit deliberate nanoparticle-cell interactions by mediating physicochemical parameters. We focus in this chapter on how nanoparticle physicochemical properties, including size, shape, elasticity, and surface modifications affect cellular uptake.

3.1. Nanoparticle size and shape

Nanoparticle physicochemical properties, such as size and shape impact nanoparticle diffusivity, surface-to-cell membrane contact area, membrane adhesion, and the strain energy required for cell plasma membrane movement [118]. In other words, nanoparticle size and shape are important physicochemical properties that determine the extent and efficiency of initial nanoparticle-cell interactions.

A study by Chithrani *et al.* reported in 2006 that 50-nm spherical gold nanoparticles exhibit highest uptake when exposed to HeLa cells in tissue culture compared to other nanoparticle sizes of 14 nm and 74 nm [119]. While the importance of nanoparticle size for cellular interaction and uptake is well established [120], reported data suggest that there are many more parameters and variables at play that affect cellular entry, such as cell phenotype, nanoparticle rate of sedimentation, density, nanoparticle morphology, and protein corona formation [14,121–124].

To illustrate how multifaceted and complex nanoparticle-cell interactions are, we highlight a study by Albanese and Chan that compared nanoparticle cell uptake of monodispersed gold nanoparticles with hydrodynamic diameters of 30 nm to 170 nm to corresponding nanoparticle aggregates [125]. The reserachers reported that HeLa and A549 cells internalized monodisperse gold nanoparticles more than the corresponding gold nanoparticle aggregates. On the other hand, a different cancer cell line (MDA-MB-435 human melanoma cells) showed an increase in nanoparticle accumulation for gold nanoparticle aggregates compared to individual monodisperse nanoparticles. This underlines the importance of cell type on nanoparticle-cell interactions and exemplifies the central role of nanoparticle size and aggregation state on cellular uptake [125].

In addition to inorganic-based nanoparticles, organic nanoparticles have also been reported to exhibit size-dependent cellular uptake patterns. In one study, different sized polymeric nanoparticles with diameters of 50 nm to 250 nm were used to probe size-dependent trends of internalization pathways in human retinal pigment epithelium (ARPE-19) cells [126]. Suen and coworkers found that smaller nanoparticles (50 nm and 120 nm in size) were internalized through clathrin- and caveolae-dependent endocytosis, while larger nanoparticles (250 nm) were internalized only *via* caveolae-mediated endocytosis. Similarly, distinct uptake patterns were observed when block copolymer nanoparticles were synthesized with different sizes using different hydrophilic chain lengths [127]. These nanoparticles formed micelles (34 nm and 49 nm in diameter) and vesicles (99 nm and 150 nm in diameter) which were incubated with WiDr (human colon carcinoma cells). Upon flow cytometry analysis, smaller micelles were internalized more quickly than the larger vesicles, but after 6 hours the cells had internalized almost equal amounts of nanoparticles for both sizes. These

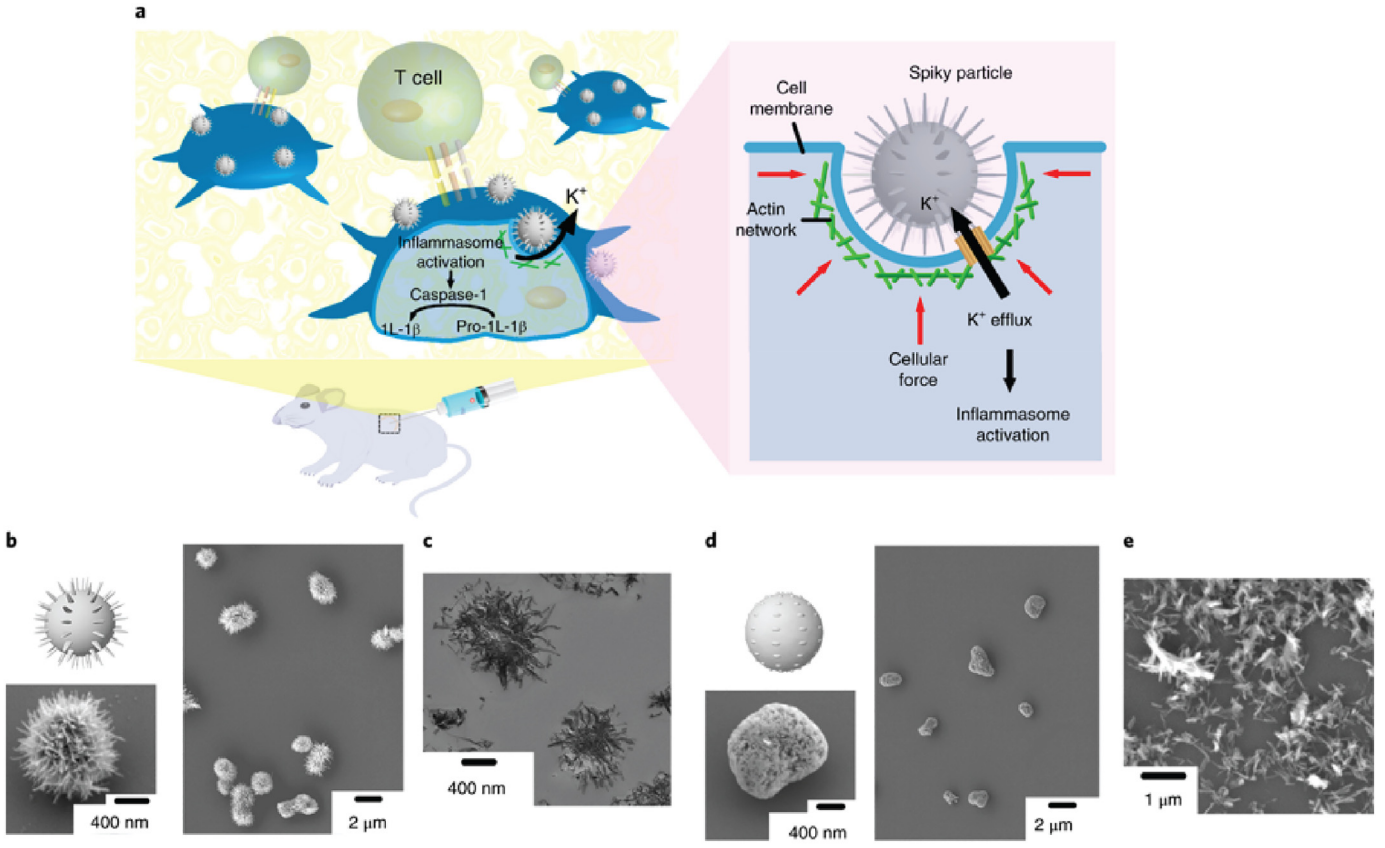

Fig. 3. Physical activation of innate immune responses *via* spiky particles. (a) Schematic illustration of the physical activation of immune cells to boost immune response *in vivo*. (b,c) Electron micrographs of spiky TiO_2 microparticles. (d) Electron micrographs of rough microparticles. (e) Electron micrograph of sonicated-off nanospikes. Reproduced with permission from Ref. [134].

studies suggest that nanoparticle size not only affects the underlying uptake kinetics but also the efficiency of nanoparticle delivery.

Besides nanoparticle size, both the shape anisotropy and orientation of the nanoparticle relative to the plasma membrane impact cellular uptake [82,128]. For example, when comparing cellular uptake between rod-like shapes and spheres, it was found by Arnida and coworkers that 50-nm spherical gold nanoparticles had higher accumulation inside human prostate cancer cells compared to gold nanorods [129]. Similarly, macropinocytosis-mediated cellular uptake of nanoparticles has been shown to exhibit a geometry-dependent correlation in both cancer and phagocytic cells.

In another study, HeLa cells and A549 cancer cells ingested rod-shaped mesoporous silica nanoparticles with varying aspect ratios *via* macropinocytosis [130]. The rod-like nanoparticles with aspect ratios between 2.1–2.5 were engulfed in higher quantities than the ones with aspect ratios between 1.5–1.7 and 4–4.5. These data suggest that cells possess mechanosensitive processes that sense slight variations in nanoparticle aspect ratio and adapt their cellular uptake response accordingly. Further data on geometry-dependent macropinocytosis concluded that primary human blood phagocytes internalize rod-like stabilized gold nanoparticles (15 nm × 50 nm) more rapidly than stabilized spherical gold nanoparticles with diameters of either 15 nm or 50 nm [131]. Nevertheless, uptake could be decreased through nanoparticle surface functionalization with 3-kDa poly(ethylene oxide). This highlights the importance of how nanoparticle surface modifications can be used as a strategy to avoid phagocytic clearance *in vivo*.

Work by Li and coworkers showed that various shapes of polymeric nanoparticles decorated with mannose surface ligands exhibited different uptake patterns and inflammatory responses in macrophages [132]. The researchers compared the uptake of mannose-decorated spherical and cylindrical micelles using RAW 264.7 macrophages. Spherical micelles were internalized through clathrin- and caveolin-dependent endocytosis and lead to a higher accumulation inside the macrophages when compared to cylindrical micelles. Interestingly, longer cylindrical micelles (215 nm × 47 nm) induced a strong inflammatory response that was linked to an increase in interleukin 6 expression [132]. As pathogenic invaders exhibit many characteristic shapes and sizes on their surfaces, immune cells may have evolved to recognize a microorganism's conserved topographic features [133].

To that end, Wang *et al.* reported that nanofeatures can participate in the activation of the innate immune system [134]. The group modified inorganic TiO_2 microparticles to bear "nanospikes" which exerted mechanical forces on innate immune cells during phagocytosis (Fig. 3). In the presence of either monophosphorylate lipid A or lipopolysaccharide only spiky particles activated K^+ efflux and inflammasomes (Fig. 3a) while the non-spiky rough particles did not (Fig. 3b, 3d). Moreover, the spiky particles along with activation of toll-like receptor 4 (TLR4) augmented dendritic cell maturation which boosted T-cell and humoral immune responses. These promising effects led to enhanced efficacy of a cancer immunotherapy and influenza vaccination *in vivo*. Ultimately, this work indicates that physical activation of immune responses through material design and particle shape (Figs. 3b–3d) could potentially be translated to other micro/nanomaterials in the future for designing more potent immunotherapies and vaccines. However, more work needs to be done to fully explore the translational potential of these results to different materials, such as polymer-based micro/nanomaterials (Fig. 3).

The examples discussed in this section highlight how nanoparticle size and shape affect cellular interactions. We anticipate that nanoparticle physicochemical properties will have significant impact on triggering and controlling robust immune responses in the future. As the field of nano-immunoengineering is still in its early stages, advanced understanding of how physicochemical properties of nanoparticles affect immune responses may lead to more potent vaccines and immunotherapies against various diseases, including cancer.

3.2. Nanoparticle charge

Researchers are able to engineer synthetic nanoparticles with positive, negative, or neutral surface charge. The nanoparticle surface charge is typically estimated by the so-called zeta potential. The zeta potential corresponds to the electrokinetic potential of a colloidal nanoparticle dispersion [135]. Experimentally, the zeta potential of nanomedicines is quantified by electrophoretic mobility measurements of colloidal dispersions in aqueous media or buffer. Therefore, a nanoparticle's zeta potential indicates the overall nanoparticle surface charge in the corresponding colloidal dispersion.

The nanoparticle zeta potential is dynamic and may change significantly in response to environmental conditions. For example, Walkey *et al.* showed in a systematic study how protein corona formation affects nanoparticle surface charge and zeta potential [15]. A library of gold and silver nanoparticles with positive, negative, and neutral surface charge were exposed to serum samples *in vitro*. Interestingly, regardless of the initial nanoparticle surface charge, the zeta potential was reported to be in the range from approximately −5 mV to −10 mV after incubation with serum proteins. These findings suggest that the formation of a serum protein corona around the nanoparticle surface leads to a "normalization" of the corresponding zeta potential. Normalization means that the adsorption of serum proteins often results in a slight overall negative surface charge of nanoparticles, regardless of their initial surface modification [15,136–142].

Since the cell plasma membrane is typically overall negatively charged, the nanoparticle zeta potential affects the likelihood of adhesion to the plasma membrane, cellular uptake, and downstream cytotoxicity. Based on Coulomb's law, cationic nanoparticles are more likely to be electrostatically attracted by the negatively charged plasma membrane resulting in increased accumulation inside cells [88,143–146]. However, several reports have demonstrated that nanoparticles with negative surface charges can also efficiently overcome the anionic cell plasma membrane and accumulate within cells [147–151]. This suggests that nanoparticle surface charge is an important design parameter when engineering nanoparticles for efficient cellular interaction, but the processes involved in nanoparticle cellular uptake are more complicated and go well beyond the simplified notion of Coulomb-driven electrostatic interactions [14,152].

While both cationic and anionic nanoparticles have been reported to enter mammalian cells, their downstream biological effects may be significantly different. As reported by Lin and coworkers, an increase in cationic charge density on nanoparticle surfaces not only promotes cellular uptake, but also elicits cytotoxic effects [153]. These adverse cytotoxic effects can be attributed to plasma membrane depolarization caused by cationic nanoparticles. This in turn can increase Ca^{2+} influx to inhibit cell proliferation [154]. Furthermore, intracellular accumulation of positively charged nanoparticles in lysosomes may result in lysosome damage, generation of reactive oxygen species (ROS), and damage of cell organelles, such as mitochondria, ultimately leading to apoptosis and cell death [155].

Other reports have demonstrated that gold nanorods displaying amine-terminated poly(ethylene oxide) promoted anti-inflammatory properties in macrophages, whereas carboxy-terminated poly(ethylene oxide) gold nanorods yielded pro-inflammatory markers [131]. *In vivo* studies have also corroborated the impact of nanoparticle surface charges on biodistribution and toxicity. Mice that were intravenously injected with positively charged lipid nanoparticles experienced severe adverse effects, including hepatotoxicity, weight loss, and a pro-inflammatory response, compared to mice injected with neutral or negatively charged nanoparticles [156]. Further studies are needed to better understand the mechanisms behind these observations and how nanoparticle surface charge affects nano-bio interactions at organ, cellular, and biomolecular levels.

When both positive and negative charges are present on a nanoparticle's surface, the resulting surface chemistry can be regarded as a

zwitterionic surface modification [157]. The generation of a zwitterionic surface modification can be advantageous and result in significant reduction of protein corona formation. This may alter and affect nanoparticle biodistribution and cellular interactions in comparison to cationic and/or anionic nanoparticle designs [158].

In summary, surface charge should be considered carefully when designing nanoparticles for biological and medical applications. We emphasize that nanoparticle surface charges are dynamic and may change over time in response to environmental and biological conditions. These changes may affect specific nano-bio interactions resulting in unintentional biological responses of nanoparticles and therapeutic outcomes of nanomedicines.

3.3. Nanoparticle elasticity

Nanoparticle elasticity can impact biodistribution, targeting, and cellular uptake [159,160]. Theoretical models of nanoparticle cellular uptake predict that plasma membrane wrapping around nanoparticles is energetically less favorable for "soft" nanoparticles than "stiff" nanoparticles. These models also indicate that soft nanoparticles have higher cellular uptake rates due to potential receptor diffusion and larger contact surface area with the cell plasma membrane [161]. Nanoparticle "softness" and "stiffness" can be quantified by a physical measure referred to as Young's modulus, which defines the relationship between stress and strain for a given material.

Due to experimental difficulties in measuring the micro- and nanoscale mechanics of nanoparticles, quantifying the effect of nanoparticle elasticity on cellular uptake is challenging. A few studies, however, report findings that emphasize the importance of nanoparticle elasticity on nanoparticle-cell interactions. Guo *et al.* have used atomic force microscopy (AFM) to measure nanoparticle elasticity and corresponding effects on nanoparticle cellular uptake [160]. In this study, authors prepared liposomes containing alginate. By changing the concentrations of calcium alginate, the cross-linking efficiency could be controlled. This control over cross-linking efficiency allowed preparation of nanoparticles with varying Young's moduli. Alginate-filled liposomes without crosslinking were "soft" (Young's modulus of ~1.6 MPa), however, increased cross-linking resulted in "stiff" liposomes with Young's moduli of up to 19 MPa. When these different nanoparticles were exposed to neoplastic cells (*e.g.*, human breast cancer MDA-MB-231 and MCF7) and non-neoplastic cells (*e.g.*, human mammary epithelial MCF10A), all cells engulfed the soft nanoparticles significantly more than the stiffer nanoparticles (*i.e.* nanoparticles with higher cross-linking efficiency and Young's modulus). Similarly, *in vivo* experiments with orthotopically implanted murine 4T1 breast cancer cells showed higher tumor accumulation of soft nanoparticles, whereas the stiffer nanoparticles were found mostly in the liver.

The elasticity of nanoparticles also affects their interaction with immune cells [159]. Anselmo *et al.* synthesized nanoparticles of the same size with elastic moduli of ~10 kPa (soft) and ~3 MPa (hard) *via* a water/PEGDA-in-oil-nanoemulsion method, where the volume fraction of PEGDA determined nanoparticle elasticity. Although *in vitro* tissue culture studies indicated lower uptake of soft nanoparticles by 4T1 murine breast cancer cells and bEnd.3 brain endothelial cells. Macrophages displayed a 3.5-fold higher uptake of hard *vs.* soft nanoparticles within 12 h of exposure. Such differences indicate shorter blood circulation times for hard nanoparticles due to increased clearance from the blood stream by phagocytotic immune cells. As the nanomedicine field continues to expand, further work dedicated to understanding of nanoparticle elasticity could improve the efficiency of nanoparticle-cell interactions.

3.4. Nanoparticle surface modifications with targeting ligands

The surface of nanoparticles can be modified with so-called targeting ligands to enable specific interaction and binding of nanoparticles to cell surface receptors. This is a prominent concept in nanomedicine and referred to as "active targeting" (Fig. 4a) [162–165]. Targeting ligands that are commonly used in nanomedicine include peptides, small molecules, proteins, antibodies, antibody fragments, and nucleic acids. We have summarized examples of nanoparticle targeting ligands in Table 2 that have been reported for *in vitro* and/or *in vivo* applications in nanomedicine. Many of these ligands are able to recognize and bind cell surface receptors that are overexpressed on malignant cells. The rationale for this surface modification strategy is that targeting ligands may increase a nanoparticle's cellular interaction, activate downstream cell signaling pathways leading to a desired biological response (*e.g.*, cell apoptosis), or enhance cellular uptake of nanoparticles to deliver therapeutic and diagnostic payloads into the cell. To engineer active targeting nanoparticles, a number of design parameters need to be taken into account and optimized for efficient targeting. These parameters include target ligand length, target ligand density, hydrophobicity, and avidity [166–173].

Nanoparticles that do not exhibit specific surface targeting ligands are referred to as "passive targeting" nanoparticles (Fig. 4b). Passive targeting indicates that the interactions between nanoparticles and cells are non-specific. These non-specific interactions may facilitate nanoparticle uptake in healthy as well as diseased cells. In contrast to active targeting nanoparticles that have not advanced beyond clinical trial stages yet, passive targeting nanoparticles have been approved by the U.S. Food and Drug Administration (FDA) as cancer nanotherapeutics, [18,174,175,180,181].

A challenge for passive and active targeting nanoparticles is that their deliberately designed surface chemistry may change upon exposure to a biological environment. For example, nanoparticles that are decorated with targeting ligands may undergo a change in their targeting abilities upon introduction into a biological milieu, such as the blood stream. One of the reasons for this is the formation of a nanoparticle protein corona due to serum protein surface adsorption. This protein corona formation changes the deliberately designed synthetic identity of nanoparticles to a biological identity which often has significant impact on nanoparticle-cell interactions [10,176,177]. Parameters that can affect formation and composition of nanoparticle protein coronae include incubation temperature, use of different protein/serum sources, human *vs.* animal plasma/serum, local temperature variations for plasmonic nanoparticles, which may facilitate the formation of unique, personalized protein coronae around nanoparticles [178]. In human plasma, nanoparticles are exposed to high amounts of protein, which increases their size and may enhance their subsequent internalization by immune cells [179]. Targeting ligands may be buried within the protein corona resulting in reduction or complete loss of specific targeting capabilities (Fig. 4c). In a study by Salvati *et al*, 50-nm silicon oxide nanoparticles were coated with transferrin, a popular protein-based ligand for active cancer cell targeting [180]. However, the ability of transferrin moieties to maintain targeting specificity diminished under physiological conditions. This was largely attributed to a shielding effect around the transferrin ligands as a result of nanoparticle protein corona formation (Figs. 4c and 4d).

To address the potential surface shielding effect of the protein corona, Tonigold and coworkers developed a pre-adsorption process to link targeting antibodies (anti-CD63) onto polystyrene carboxy-functionalized nanoparticles [181]. Antibodies that simply adsorbed to the nanoparticle surface were compared with the antibodies that were coupled *via* 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC-NHS) chemistry. Regardless of how the antibodies adhered to the nanoparticle surface, the constructs had maintained similar properties in the absence of serum and were able to bind to CD63 antigen expressed on monocyte-derived dendritic cells. However, under physiological conditions (*e.g.,* in plasma or serum), an impaired targeting efficiency correlated to the method of antibody attachment. The authors observed that in 100% emersion of serum or plasma, the nanoparticles that are covalently bound to the antibodies

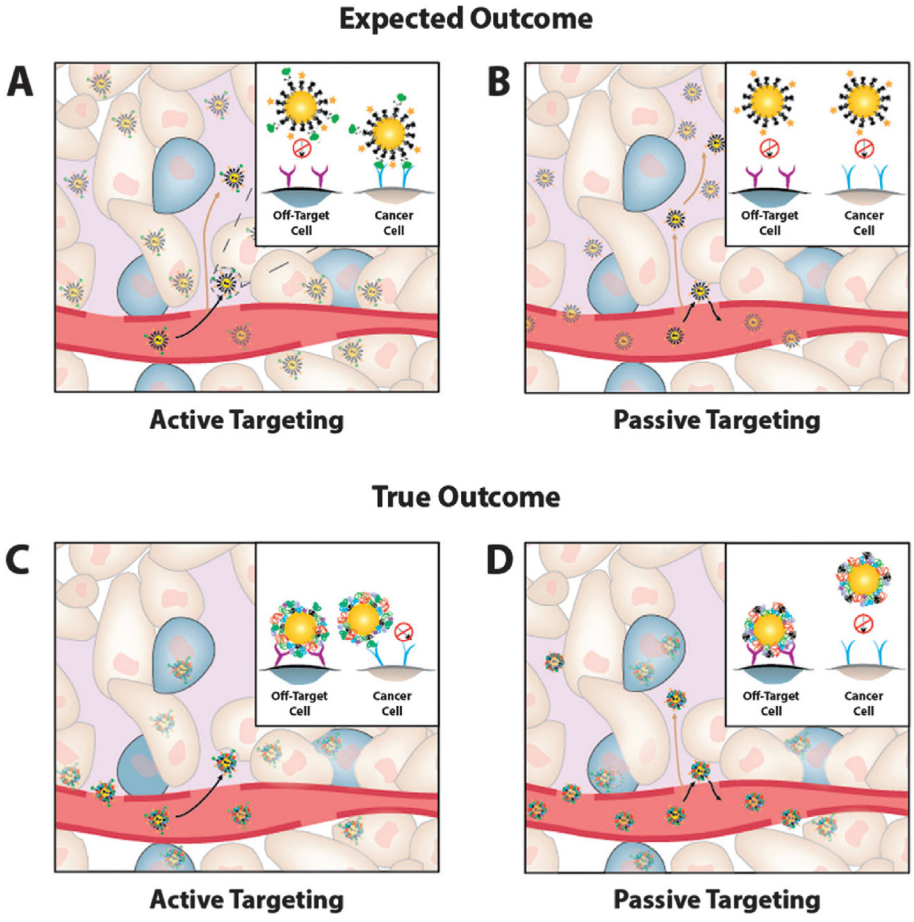

Fig. 4. Protein corona formation can affect intratumoral cell targeting abilities of active and passive targeting nanoparticles. (A) Upon extravasation from tumor blood vessels into the tumor interstitium, active targeting nanoparticles are designed to specifically recognize and bind cell surface receptors on malignant cells in the tumor microenvironment. This phenomenon is in contrast to passive targeting nanoparticles shown in (B) that do not exhibit specific cell targeting capabilities. (C) Upon exposure to a biological environment, the protein corona formation may sterically hinder and mask surface-bound ligands of active targeting ligands to facilitate non-specific interactions with off-target cells rather than targeted cancer cells. (D) Such non-specific cellular interactions with off-target cells can also be observed for passive targeting nanoparticles. Reproduced with permission from Ref. [66].

lost their targeting abilities, whereas the pre-absorbed antibody-nanoparticles were still able to efficiently target the CD63 antigen on monocyte-derived dendritic cells.

A different approach to mitigate the negative impact of the protein corona on nanoparticle cell targeting was reported by Dai *et al.* in 2014 and is referred to as poly(ethylene glycol) (PEG) backfilling [67]. Authors decorated gold nanoparticle surfaces with PEG molecules of different molecular weights and covalently conjugated these PEG molecules with trastuzumab, a monoclonal ErbB2 (HER2) targeting antibody. They concluded that the PEG chain length used to conjugate the targeting antibody affects nanoparticle targeting efficiency. In more detail, the PEG molecules used for surface backfilling need to be of lower molecular weight than the PEG molecules used to conjugate the targeting antibody. This backfilling strategy enabled specific targeting of nanoparticle surface conjugated trastuzumab antibodies to ErbB2 cell surface receptors. In addition, Dai *et al.* demonstrated that surface backfilling with low molecular weight PEG reduced the formation of a serum protein corona. Overall, this design improved targeting specificity of nanoparticles to ErbB2 expressing cells in serum-rich environments in comparison to other surface modification strategies.

While the benefit of active targeting has been demonstrated for *in vitro* tissue culture studies through a large body of publications, it is less clear if the presence of active targeting ligands on nanoparticle surfaces can result in increased specific cellular interaction *in vivo*. For example, a study by Chan and coworkers published in 2018 demonstrated that there was no statistically significant difference in nanoparticle-cell interaction for 55-nm gold nanoparticles with and without ErbB2 targeting trastuzumab antibody surface modification in preclinical mouse models of human ovarian cancer SKOV-3 xenograft tumors [17]. In addition, this study also demonstrated that intratumoral nanoparticles irrespective of surface modification are more likely to interact with tumor associated macrophages (TAMs) rather than targeted malignant cells. This finding is in line with reports by Weissleder and coworkers, which identified TAMs as major intratumoral biological barriers for targeted nanoparticle delivery to cancer cells [182–184].

Decorating nanoparticle surfaces with targeting ligands that are specific against cell surface receptors may increase cellular interactions. However, surface modification strategies need to be chosen judiciously and optimized for intended applications. Precise targeting of cell populations *in vitro* and *in vivo* requires that nanoparticle surface modifications can maintain their deliberately designed functions in dynamically changing biological environments. These findings open opportunities for researchers to develop nanoparticle surface designs that can address the above-mentioned challenges to improve specific delivery and interactions between nanoparticles and targeted cells.

In this chapter, we surveyed and discussed a number of nanoparticle physicochemical properties that are critical for enhanced nanoparticle-cell interactions. Nanoparticle size, shape, surface charge, elasticity, and surface ligands and ligand density are important design criteria that researchers need to consider when engineering nanomedicines for cellular interaction and uptake. After successful cellular entry, nanoparticles need to overcome intracellular barriers to reach cellular compartments

Table 2
Examples of different types of nanoparticle targeting ligands for enhanced nanoparticle cellular interaction with targeted cell types.

Targeting ligand	Nanoparticle core material	Targeted cell types	Used for cell targeting *in vitro* or *in vivo*	Ref.
Peptides				
CLT1 peptide for fibronectins	PEG-PLA	C6 glioma cells	*In vitro* and *in vivo*	[314]
CREKA pentapeptide sequence for fibrin	DSPE-PEG_{2000}	GL261 glioma cells	*In vivo*	[315]
M2-macrophage targeting peptide	HPMA polymer	Tumor associated macrophages	*In vitro* and *in vivo*	[316,317]
RGD motif for integrin binding	Mesoporous silica	SCC-7 mouse squamous cell carcinoma, HT-29 human colon cancer cells	*In vitro*	[318]
P160 targeting peptide found through phage display	Cadmium-selenide core zinc-sulfide shell quantum dots	MCF-7 human breast cancer cells	*In vitro*	[319]
Chlorotoxin peptide for MMP2	Silver	U87MG glioblastoma	*In vitro* and *in vivo*	[320]
Glycoproteins				
Transferrin to cross blood brain barrier	DSPC-cholesterol-POPG	U87MG glioblastoma, GL261 glioma cells	*In vitro* and *in vivo*	[321]
Antibodies and antibody fragments				
Anti-CD8a $F(ab')_2$	PLGA-PEG	$CD8^+$ T-cells	*In vitro* and *in vivo*	[322]
HuA33 monoclonal antibody	Poly(methacrylic) acid	LIM1889, LIM2405+, LIM2405-human colon cancer cells	*In vitro*	[323]
HER2 monoclonal antibody	Gold plasmonic vesicles	SKBR-3 human breast cancers	*In vitro*	[324]
Anti-epidermal growth factor receptor	Quantum dots	MDA-MB-231 mammary adenocarcinoma, BxPC-3 pancreatic adenocarcinoma	*In vitro*	[325]
Nucleic acids				
Single-stranded oligonucleotide-based aptamers	Quantum dots	A549 lung adenocarcinoma	*In vitro*	[326]
Anti-cMet DNA aptamer	Lipidated aptamer-based nanocarriers loaded with doxorubicin	H1838 non-small cell lung	*In vitro*	[327]
G- rich DNA aptamer	Zinc gallogermanate	4T1 mammary carcinoma	*In vivo*	[328]
Small molecules				
Folic acid for folate receptors	Mesoporous silica	U20 osteosarcoma	*In vitro*	[329]

Abbreviations: CTL1: fibronectin targeting peptide, CREKA: fibrin binding peptide cysteine, arginine, glutamic acid, lysine, aspartic acid, RGD: arginine, glycine aspartic acid, MMP2- Matrix metalloproteinase, PEG-PLA: poly(ethylene glycol)-poly(lactide), DSPE-PEG2000: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000], HPMA: poly (N-(2-hydroxypropyl) methacrylamide), DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine, POPG: 1,2-distearoyl-sn-glycero-3-phosphocholine, PLGA-PEG: poly(lactic-co-glycolic acid) (PLGA) and polyethylene glycol

and organelles. We explore and discuss the intracellular journey of nanoparticles in the next chapter.

4. Intracellular trafficking of nanoparticles

After cellular internalization, nanoparticles undergo transport and trafficking to various intracellular destinations. If nanoparticle cellular uptake occurs *via* endocytic pathways, nanoparticles are confined within a membrane-lined vesicle, such as an endosome (Fig. 1). These vesicles transport throughout the cell in complex trafficking patterns. Currently used methods for probing the intracellular trafficking of nanoparticles include optical- and electron-based microscopy techniques, such as super resolution fluorescence microscopy, confocal laser scanning microscopy, dark-field microscopy, transmission electron microscopy, scanning electron microscopy, atomic force microscopy, flow cytometry, mass cytometry, photoacoustic microscopy, surface-enhanced Raman scattering, laser-ablation inductively-coupled plasma mass spectrometry, and correlative microscopy [185–187].

Due to the complexity of nanoparticle intracellular trafficking patterns, it is challenging to paint a complete picture of all intracellular events and processes that take place once nanoparticles enter cells. Hence, we present in Fig. 5 a simplified overview of intracellular nanoparticle transport processes and refer readers to excellent specialized overview articles on this topic [188–190].

Similar to nanoparticle cellular uptake (see Chapters 2 and 3), nanoparticle intracellular trafficking is also dependent upon cell type and a nanoparticle's physicochemical properties, including size, shape, and surface chemistry.

To briefly review the dynamics of intracellular nanoparticle transport, we highlight findings reported by Al-Hajaj and coworkers. Using *in vitro* tissue culture experiments, the researchers compared differences of nanoparticle trafficking in liver cancer cells and non-malignant kidney cells [191]. In this study, researchers modified the surfaces of semiconductor (quantum dots) nanoparticles made from CdSe@CdZnS with sizes of 8-10 nm and four different surface chemistries: (i) mercaptopropionic acid, (ii) dihydrolipoic acid, (iii) L-cysteine, or (iv) cysteamine ligands. The overall size of quantum dots was not affected by these different surface chemistries. Interestingly, the highest cellular uptake in both liver and kidney cell lines was reported for quantum dots modified with cysteamine, potentially due to their overall cationic surface charge. However, after cellular uptake of these nanoparticles, p-glycoprotein transporters were shown to excrete between 60-70% of the initially accumulated quantum dots with cysteamine surface chemistry in both cell lines over the course of 6 hours. These data demonstrate that intracellular nanoparticles may be excreted from cells over time due to dynamic intracellular transport and trafficking processes. While these data have been obtained in tissue culture experiments, this information is important as liver and kidney cells are involved in degradation, metabolism, and elimination of administered nanoparticles, which are prime aspects of *in vivo* nanotoxicology [192–195].

In this chapter, we explore how nanoparticles can be rationally designed to overcome intracellular barriers using endosomal escape

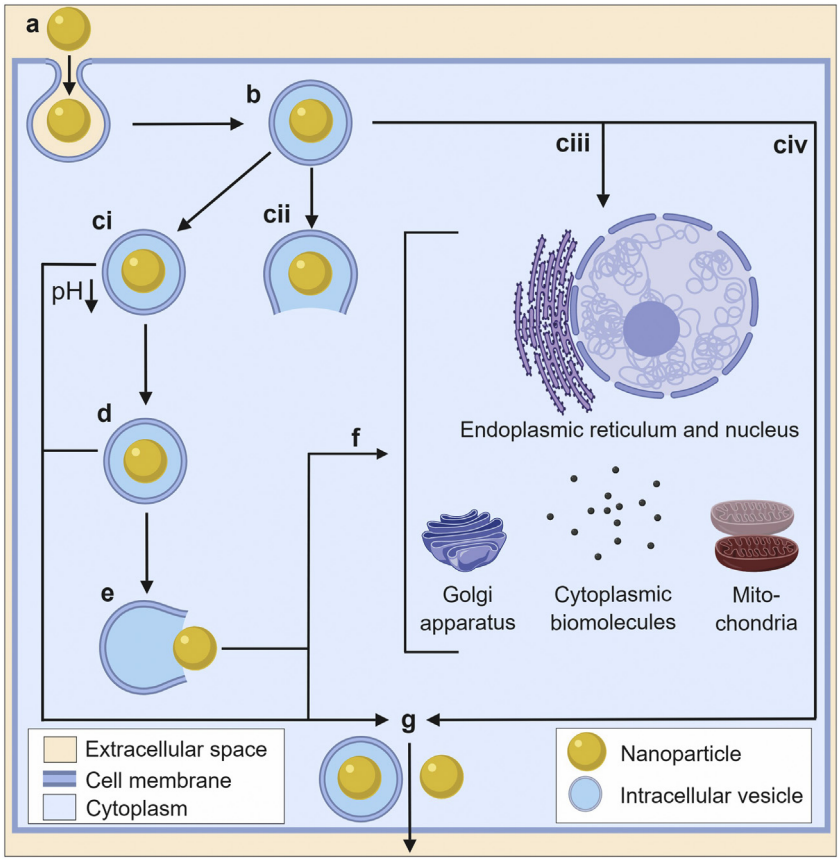

Fig. 5. Simplified overview of main nanoparticle intracellular trafficking pathways and mechanisms. (a) Cellular entry of nanoparticles often occurs through an endocytotic pathway. (b) After internalization, nanoparticles are typically trafficked to a sorting/early endosome which can undertake several intracellular transformations. (ci) The sorting/early endosome forms a late endosome as the pH decreases that then fuses with other components to become a lysosome (d) from which nanoparticles can either escape for subsequent intracellular targeting (f) or in which nanoparticles can get degraded and/or exocytosed (g). An alternative to lysosome formation can either be (cii) endosomal escape and subsequent intracellular targeting (f), or (ciii) a recycling process through a recycling endosome located near the perinuclear region, or even (civ/g) rapid recycling directly to the plasma membrane.

mechanisms. We then explore and discuss nanoparticle intracellular organelle targeting and finish with an overview on cellular excretion processes.

4.1. Endosomal escape

Unless interrupted, cells process endocytosed nanoparticles in similar ways as internalized biomolecules (*e.g.,* proteins, carbohydrates, nucleic acids, and lipids). Upon endocytosis, nanoparticles are typically entrapped in vesicular structures, such as endosomes (Figs. 5a and 5b). Endosomes can undergo a variety of processes such as vesicle aging, that can be briefly characterized by early- to late-stage vesicle transformation (Figs. 5b– 5d). These different stages of intracellular vesicle development are typically accompanied by changes in intra-vesicle pH (Fig. 5 ci). Eventually, the acidified endosome may fuse with lysosomal compartments for enzymatic digestion and degradation of vesicle contents (Fig. 5d). Examples for these pathways are the formation of phagolysosomes and macropinosomes [39,196]. Endosomes can also be recycled and/or processed at the perinuclear region (Fig. 5 ciii). Viruses often exploit trafficking patterns that lead to the perinuclear region for pathogenesis [197,198]. Endosomes can also be sent to the plasma membrane for downstream exocytosis (Fig. 5 civ). Pridgen *et al*. capitalized on this pathway for the transepithelial transport of nanoparticles [199]. Nanoparticles bearing Fc regions of the IgG antibody were shown to target the neonatal Fc receptor which resulted in enhanced absorption efficiency after oral administration *in vivo*.

More often than not, endosomal entrapment represents a major detriment to nanomedicine efforts. Once confined to intracellular vesicles, nanoparticles may be subjected to lysosomal degradation which can inhibit their intended biological and therapeutic functions. In consequence, some types of nanoparticles need to overcome the endosomal barrier before accessing the cell's cytoplasm, intracellular organelles, and compartments (Figs. 5e- 5f). Physicochemical nanoparticle properties, such as surface charge and surface ligand display, can be engineered to facilitate endosomal escape with enhanced efficiency (Figs. 5 cii, 6).

Strategies for endosomal escape of nanoparticles include the use of CPPs and other membrane disrupting nanoparticle surface modifications and mechanisms, such as lipid fusion with the endosomal membrane (Fig. 6a) [105]. In recent studies, nanoparticles decorated with CPPs have been reported to result in endosomal rupture and escape of nanoparticles *via* the so-called "proton sponge" effect [200,201]. Although not fully understood, the proton sponge effect has been attributed to cationic surface modifications which induce osmotic swelling and subsequent membrane disruption to release endosomal contents, such as nanoparticles and their payloads.

Dalal and coworkers coated quantum dots in a polyacrylate shell that was further modified with PEG and varying amounts of TAT peptides [202]. These peptides are derived from transactivator of transcription (TAT) of the human immunodeficiency virus and are typical examples for CPPs. As reported by Dalal *et al*., nanoparticles with TAT peptide surface modification entered HeLa cells through the lipid-raft mediated endocytotic pathway. Peptide-modified quantum dots were shown to have diverse trafficking patterns as a function of the CPPs' multivalency (number of attached peptides per nanoparticle). Lower multivalency of TAT peptides on the quantum dot surface resulted in endosomal escape and localization of nanoparticles at the Golgi apparatus and the perinuclear region as documented by fluorescence

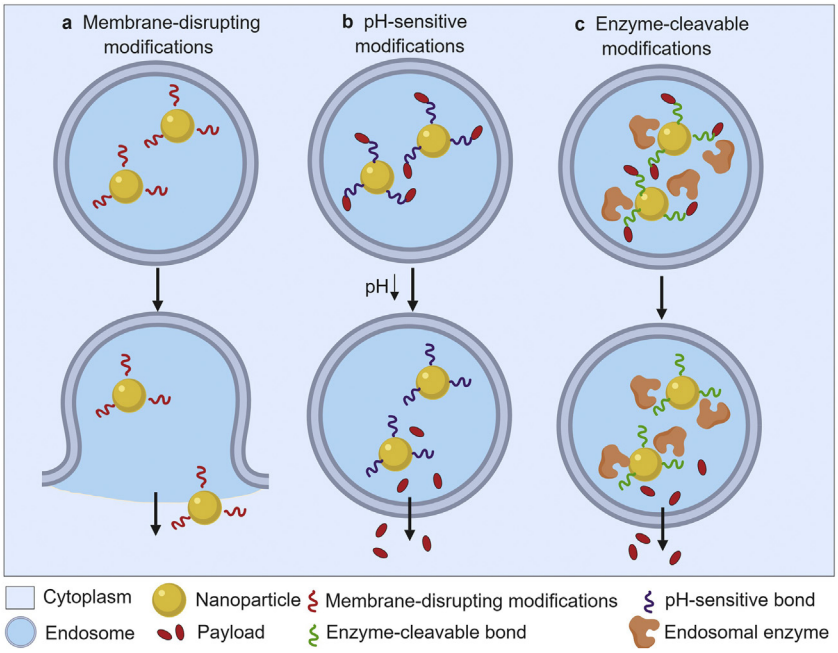

Fig. 6. Examples of cytoplasmic delivery *via* endosomal escape. Three main strategies are available for nanoparticles to break through and escape endosomal barriers. (a) Membrane-disrupting surface modifications and mechanisms (*e.g.*, poly(ethyleneimine) PEI; cell-penetrating peptides (CPPs); and lipid fusion with endosomal membrane); (b) pH-responsive materials (*e.g.*, hydrazone bonds); and (c) enzyme-cleavable materials (*e.g.*, ester linkages, cathepsin B cleavable peptides).

microscopy. In contrast, higher multivalency of CPPs on the quantum dot surface led to exocytosis of nanoparticles. Similar targeting results were obtained with the same CPP sequence on mesoporous silica nanoparticles rather than quantum dot nanoparticles [203]. This underlines the importance of the specific amino acid sequence of CPPs of different types of nanoparticles to regulate escape and targeting mechanisms.

In addition to membrane-destabilizing peptides, other membrane-disrupting surface modifications have been utilized (Fig. 6a). Nanoparticles modified with polymers, such as poly(ethyleneimine) (PEI), have been reported to rupture endosomes [204]. Melamed and coworkers capitalized on PEI's ability to disrupt endosomal membranes to deliver siRNA *via* spherical nucleic acids that sensitized glioblastomas to a chemotherapeutic agent. The researchers observed decreases in proliferation and metabolism, as well as an increase of senescence in the glioblastomas.

An alternative pathway for endosomal escape that is not depicted in Fig. 6a has been reported in a recent study by the Irvine group at MIT. First, they fabricated 2-4 nm amphiphilic gold nanoparticles that embedded a TGF-β inhibitor [205]. Next, the nanoparticles were coated with antibodies to target $CD8^+$ T-cells *in vitro* and *in vivo*. The amphiphilic nanoparticles displayed the ability to directly translocate across the cell membrane. Interestingly, transmission electron microscopy (TEM) analysis revealed 24 hours post-incubation *in vitro* that the amphiphilic nanoparticles traversed endosomal membranes to deliver the TGF-β inhibitor payloads into the cytoplasm of $CD8^+$ T-cells. The authors concluded that the targeting antibody was proteolytically degraded in the endolysosomal pathway which liberated the amphiphilic nanoparticles to penetrate through the intracellular vesicles' membranes *via* non-disruptive membrane penetration. *In vivo*, the unique nanoparticle-cell behavior correlated with 40-fold enhanced $CD8^+$ T-cell nanoparticle uptake. The improved nanoparticle uptake resulted in greater cytokine production due to the enhanced TGF-β inhibitor delivery. This study elegantly illustrates the potential impact of how surface modifications can simultaneously facilitate specific cell uptake and escape from intracellular vesicles.

A key characteristic of intracellular endosomes and lysosomes is their acidic pH which can be exploited for endosomal escape of pH-sensitive nanoparticle modifications (Fig. 6b). Wang *et al.* harnessed the acidic endosomal pH environment to deliver the cancer drug doxorubicin to multidrug resistant cancer cells [206]. The researchers bound doxorubicin to 30-nm diameter gold nanoparticles through a low pH-sensitive hydrazone bond. Once the nanoparticles entered the multidrug resistant cells through clathrin- and caveolae-dependent endocytosis, the hydrazone bonds were cleaved due to the acidic pH of the late stage endosomes/lysosomes which released doxorubicin from the gold nanoparticle core into the cytoplasm. Flow cytometry analysis and confocal microscopy were used to confirm cytoplasmic doxorubicin concentrations. Furthermore, the authors were able to overcome doxorubicin efflux caused by P-gp transporter pumps by using this hydrazone-doxorubicin gold nanoparticle system. High levels of doxorubicin were maintained within drug resistant cells, leading to a therapeutic effect in comparison to free unbound doxorubicin.

To further highlight the utility of pH-sensitive nanoparticles (Fig. 6b), Farokhzad's group grafted poly(2-(diisopropylamino ethylmethacrylate) into the amphiphilic polymer coating of nanoparticles displaying a tumor targeting peptide sequence (*i.e.*, RGD sequence) [207]. Once trapped in endosomes, these nanoparticles underwent rapid protonation. The pK_a of poly(2-(diisopropylamino ethylmethacrylate) is close to endosomal pH (*i.e.*, pH 6.0-6.5), which allowed the nanoparticles to efficiently release siRNA payloads upon endosomal acidification. Using this siRNA delivery strategy, optical microscopy confirmed the efficient knockdown of luciferase in luciferase expressing HeLa cells. These examples demonstrate that nanoparticles can be designed effectively to exploit the chemical characteristics of intracellular vesicles, such as acidic pH, for improved cytoplasmic delivery of payloads, including nucleic acids (Fig. 6b).

Enzyme-cleavable nanoparticle modifications, such as linkers and shells, are a third strategy for endosomal escape (Fig. 6c). These moieties have been used to enable cytoplasmic delivery of nanoparticles and nanoparticle payloads trapped in endosomal vesicles. For instance, Prasetyanto *et al.* concealed cytotoxic proteins inside enzyme-breakable disulfide-linked organosilica nanoparticles capsules which were exposed to a rat glioma cancer cell line *in vitro* [208]. Seven minutes after incubation, scanning electron microscopy revealed that the glioma cells had already internalized the enzyme-breakable nanoparticles. Once inside the glioma cells, colocalization of these nanoparticles was

observed with intracellular lysosomes leading to subsequent enzyme-mediated nanoparticle degradation and release of cytotoxic cargo. Forty percent of the glioma cells were viable after being exposed to this stimuli-responsive nanoparticle system. After 48 hours, the enzyme-breakable organosilica nanocapsules were entirely degraded within lysosomes.

Similarly, Sangtani *et al.* synthesized ~35-nm in diameter quantum dots conjugated to both CPPs and doxorubicin through various enzyme-cleavable linkers [209]. The quantum dot-CPP conjugates bound to doxorubicin *via* ester linkages were reported to have been enzymatically cleaved within HeLa cells with highest efficiency inside endosomes. This degradation resulted in a cluster of doxorubicin fluorescence signal in the HeLa cells' nuclei. When assessing cytotoxicity, the QD-CPP-ester-doxorubicin conjugates were reported to achieve doxorubicin delivery to the nucleus in two hours.

Additional enzymatic studies reported by Acar *et al.* have identified and exploited endosome-specific enzymes like cathepsin B to release cargo from amphiphilic nanoparticles upon endosomal entrapment [210]. Endosome-specific enzyme-cleavable linkers were added between the hydrophilic peptide payload and the hydrophobic lipid with the conjugation of fluorescence resonance energy transfer (FRET)-coupled dyes on each side of the molecule. This FRET-based approach enabled the tracking of the lipid-conjugated peptide-based nanoparticles as well as the cleavage kinetics through confocal microscopy. Interestingly, after the enzymatic cleavage, the hydrophobic lipid part of the structure exited the cytoplasm through extracellular vesicles. As demonstrated by this example, nanoparticles can be engineered to allow tracking of their intracellular transport pathways and the fate of their individual molecular components. These findings not only further our understanding of intracellular nanoparticle trafficking but also facilitate elucidation of the intracellular fate of nanoparticle building blocks and payloads. However, more work is needed to achieve finite control over such stimuli-responsive nanoparticle systems *in vivo*.

The examples presented in this section highlight that different intracellular nanoparticle delivery patterns and efficiencies can be achieved through careful engineering of nanoparticle surface chemistry.

4.2. Organelle and subcellular targeting

As shown in Fig. 5, mammalian cells exhibit a variety of different intracellular organelles and compartments. Organelles, such as the cell nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus exert specific biological functions that are important for replication, cell division, energy production, lipid and protein synthesis, and intracellular transport. While these functions are strongly regulated, conserved, and concerted in healthy cells, organelle dysfunction, alteration, and deregulation of intracellular mechanisms represent typical hallmarks of disease. Therefore, targeting intracellular organelles and compartments with nanoparticles is important for diagnosis and therapy.

Similar to nanoparticle uptake and endosomal escape, engineering physicochemical properties of nanoparticles can facilitate transport to specific organelles. While targeting moieties can aid in organelle localization (see Table 3), there are other important factors to successful nanoparticle delivery. For instance, several studies have shown a size-dependent relationship for nanoparticle transport into the cell nucleus for therapeutic interventions [211–214]. As shown in Fig. 7, gold nanoparticles with an average diameter of ~4 nm were able to enter the cell nucleus of MDA-MB-231 human breast cancer cells, while gold nanoparticles with a diameter of approximately 14 nm were not able to cross the nuclear envelope and remained distributed throughout the cell's cytoplasm.

Nanoparticle entry into the cell nucleus has been reported by two different mechanisms. The first one is active transport of nanoparticles through the nuclear membrane pore complex that is facilitated by nuclear localization signals (NLS). Typical examples of these NLS sequences are short peptides that can bind to cytoplasmic importins, such as Importin α and Importin β, that are located in the perinuclear region [212]. After binding to cytoplasmic importins, nanoparticles with diameters up to 50 nm have been reported to enter the cell nucleus *via* active transport through the nuclear pore complex [203]. A second pathway of nanoparticle entry into the cell nucleus is by passive diffusion of cytoplasmic nanoparticles through the open channel of the nuclear pore complex. Diameters of this channel can vary, but are typically in a range of ~6−9 nm [211–214]. Passive diffusion into the cell nucleus requires that nanoparticles are smaller in diameter than the cut-off size of the nuclear pore complex.

Recently, García *et al.* reported enhanced cellular uptake of functionalized 14-nm cationic gold nanoparticles when compared to 2-nm and 5-nm diameter gold nanoparticles [215]. Despite better cellular uptake for the larger nanoparticles, the 2-nm nanoparticles delivered DNA payloads to the nucleus 20x times more efficiently than their 14 nm counterparts. Another study by Huo and coworkers determined a size-dependent basis for nuclear uptake of gold nanoparticles in breast cancer cells (MCF-7) [216]. Nanoparticles with diameters of 2 nm and 6 nm were seen inside the nucleus while larger nanoparticles of 10 nm and 16 nm were not. Building upon that observation, the researchers used the 2-nm gold nanoparticles to deliver triplex-forming oligonucleotides to the nucleus which silenced the c-myc promoter by 50%. Work by Oh and coworkers also support the concept of size-dependent nanoparticle delivery to the cell nucleus, as reported for gold nanoparticles modified with PEG and polyarginine [217]. The researchers observed nucleus targeting abilities for smaller nanoparticles (~2.4 nm in diameter) while slightly larger nanoparticles (5.5–8.2 nm) were distributed in the cytoplasm. Not only are size and surface chemistry important for nuclear localization, but as Tang *et al.* showed nuclear transport of quantum dots plateaued when the quantum dot surface was covered at 20% density with NLS sequences [113]. Moreover, Tang *et al.* also confirmed a size dependence for cell uptake and nuclear internalization using semiconductor quantum dot nanoparticles.

Mitochondria are also major intracellular targets for nanoparticles and therapeutic payloads. In one study, Qu and colleagues coupled triphenoylphosphonium (PPh_3) to the surface of doxorubicin-loaded mesoporous silica nanoparticles (MSNPs-PPh_3-DOX) [218]. As shown by the authors, the lipophilic nature of the three phenyl groups in combination with the delocalized cationic charge of this surface ligand facilitated nanoparticle transport across the mitochondrial membrane.

Table 3
Nanoparticle Surface Modifications and Surface Ligands for Intracellular Organelle Targeting.

Nanoparticle Surface Modification	Organelle Destination (s)	Ref.
Folic acid	Nucleus	[329]
RGD + CGGGPKKKRKVGG peptide	Nucleus	[330]
3,4-diphenylacetic acid-MYIEALDKYAC-COOH peptide	Nucleus	[331]
DRQIKIWFQNRRMKWKK peptide	Nucleus	[332]
Triamcinolone acetonide	Nucleus	[333]
Nuclear localization sequence (NLS) peptide	Nucleus	[334]
Triphenoylphosphonium	Mitochondria	[218–220]
$D[KLAKLAK]_2$ peptide	Mitochondria	[335]
RGD + MLALLGWWWFFSRKKC peptide	Mitochondria	[336]
MVSGSSGLAAARLLSRTFLLQQNGIRHGSYC peptide	Mitochondria	[337]
Dmt-D-Arg-Phe-Lys-NH2 peptide	Mitochondria	[338]
Octaarginine peptide	Golgi apparatus, mitochondria	[49], [339]
TAT peptide CALNNAGRKKRRQRRR	Golgi apparatus, nucleus	[245], [202]
L-cysteine moieties	Golgi apparatus	[340]
KDEL peptide	Endoplasmic reticulum	[341]
mi-R29b + PEI	Endoplasmic reticulum	[342]

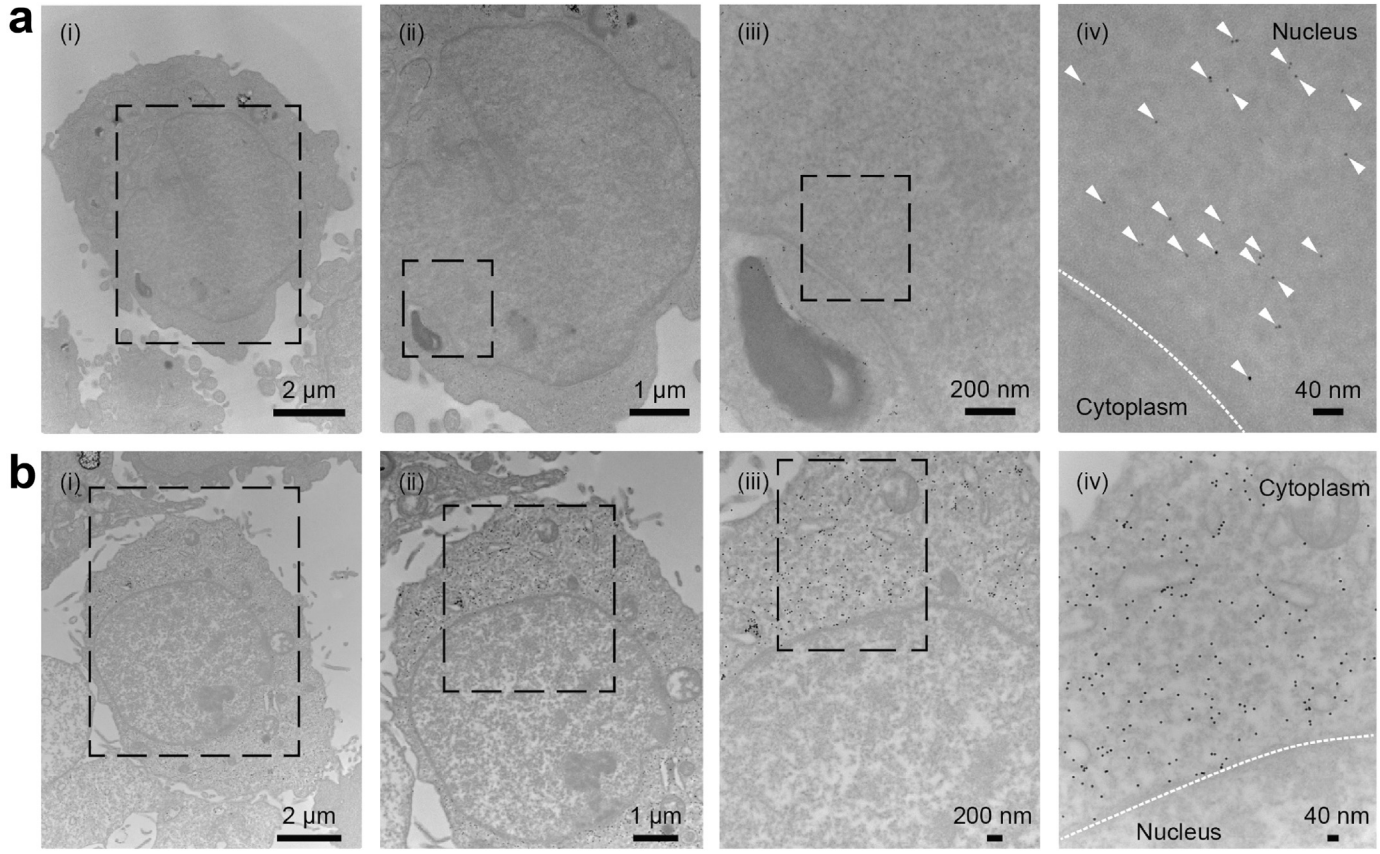

Fig. 7. Size-dependent transport of gold nanoparticles into cell nucleus of MDA-MB-231 human breast cancer cells in tissue culture. Cells were exposed to gold nanoparticles modified with cationic peptide sequences. Gold nanoparticles exhibited two different sizes: (a) 4-nm; and (b) 14-nm. Cells were exposed to nanoparticles at a concentration of 4 μM for 24 h in tissue culture. Transmission electron microscopy (TEM) images of 4-nm gold nanoparticles with increasing magnification (a,i – a,iv) reveal that nanoparticles are homogenously distributed throughout the cell cytoplasm and were able to enter the cell nucleus (a,iv). White arrowheads indicate 4-nm gold nanoparticles within cell nucleus. Gold nanoparticles with a diameter of 14-nm were also distributed throughout the cell cytoplasm (b,i – b,iii), but remained within the cytoplasm and were not able to cross the nuclear envelop (b,iv). Black dashed rectangles indicate area of magnification of subsequent image. The white dashed lines indicate the nuclear membrane. These data suggest a size-dependence for nuclear transport of nanoparticles *via* passive diffusion through the nuclear pore complex.

Within 8 hours, MSNPs-PPh_3-DOX had been released from lysosomes and interacted with the mitochondria. This resulted in reduced cellular levels of ATP in HeLa cells and caused mitochondrial dysfunction which lead to a reduction in cell viability to only 30% after 24 hours of *in vitro* exposure.

Mitochondria dysfunction is a key player in neurodegenerative diseases including Alzheimer's disease. To that end, Kwon *et al.* developed a cerium oxide nanoparticle-based system that could suppress the onset of neuron death by sequestering reactive oxygen species generated by malfunctioning mitochondria [219]. The researchers found that cerium oxide nanoparticles coated with DSPE-PEG_{2000}-methoxy and PPh_3 could recycle oxygen atoms and inhibit further neuronal damage *in vivo*.

Building upon mitochondria targeting with nanoparticles, Jeena *et al.* synthesized novel peptide amphiphiles that were fine-tuned to undergo self-assembly upon accumulation in mitochondria [220]. In HeLa cells, amphiphilic peptides consisting of β-sheet forming blocks conjugated to PPh_3 were seen to accumulate in mitochondria that then formed fibrils due to high local concentration. The formation of the fibrils perturbed mitochondrial membranes leading to the leaking of mitochondrial contents into the cytoplasm and subsequent apoptosis. Future work using this construct would require specific cell precision so as not to induce mitochondrial damage in healthy cells.

The endoplasmic reticulum is an important cellular organelle for the proper folding and transport of proteins [221]. Moreover, the endoplasmic reticulum is a critical site for the loading of peptides into MHC class I molecules and subsequent cytotoxic T-cell responses [222]. Therefore, endoplasmic reticulum integrity is critical for proper cell and organism functioning. Cubillos-Ruiz *et al.* illustrated how endoplasmic reticulum stress in tumor-associated dendritic cells promotes tumor growth and dampens anti-cancer immunity [223]. Identification of an increase in endoplasmic reticulum stress factor (XBP1) was linked to reduced dendritic activity which also reduced T cell dependent immunity. By employing PEI-based nanoparticles that encapsulated specific siRNAs, the phagocytic dendritic cells were seen to preferentially engulf these nanocomplexes which induced about 65% gene silencing of XBP1. Silencing of endoplasmic reticulum stress in tumor associated dendritic cells through intraperitoneal injections of the siRNA-PEI nanoparticles *in vivo* augmented the survival of mice with aggressive orthotopic ovarian tumors. This novel method provides a way to activate cell-mediated anti-cancer immunity through precise intracellular nanoparticle targeting.

Another important organelle for nanoparticle-based interventions is the Golgi apparatus. This organelle is critical for carrying out posttranslational modifications of newly synthesized proteins [224]. Since accumulations of misfolded proteins are a hallmark of cancerous cells, Yu *et al.* sought to inhibit the Golgi apparatus' pathological role [225]. To do this, the researchers first encapsulated both a COX-2 inhibitor and Brefeldin A into PLGA-PEG nanoparticles. The COX-2 inhibitor, (celecoxib) accumulated within the Golgi apparatus while Brefeldin inhibited protein transport from the endoplasmic reticulum to the Golgi apparatus. Fluorescence microscopy revealed that the nanoparticle encapsulation of these two small molecules efficiently damaged the Golgi apparatus within 30 minutes *in vitro* in murine metastatic breast cancer cells and displayed enhanced cytotoxicity. Furthermore, the co-delivery of these small molecules was observed to decrease the expression of proteins associated with metastasis (MMP-9 and VEGF).

Genetic material encapsulated into nanoparticles must also overcome endosomal barriers to reach cytosolic targets. To that end, Tai and colleagues produced 30-nm self-assembling ribonucleoprotein-octamer nanoparticles that contained 38% of siRNA by weight without excessive cationic charges [226]. Efficient endosomal escape was achieved *via* incorporation of a histidine-rich peptide sequence into the nanoparticle architecture that resulted in protonation, osmotic swelling, and subsequent cargo release. *In vivo*, the octamer extended survival of mice through efficient and targeted knockdown of cytoplasmic polo-like kinases in prostate cancer mouse models thus confirming the success of this new system as a potential therapeutic.

Efficient nanoparticle-mediated co-delivery of molecules to specific intracellular targets is a promising facet in nanomedicine. The studies highlighted in this section are examples of current strategies for intracellular organelle targeting in the nanomedicine field and underline the potency of efficient and effective nanoparticle delivery to specific intracellular organelles. In Table 3, we provide an overview of nanoparticles surface modifications and surface ligands that are commonly employed for nanoparticle organelle targeting.

Therapeutic and diagnostic success of nanomedicines often hinges on efficient intracellular transport of nanoparticles. While organelle targeting is a powerful approach to increase the potency of nanomedicines, further studies to improve endosomal escape and delivery of nanoparticles and their payloads to intracellular targets are needed. Successful organelle targeting *in vivo* proves to be a formidable challenge. First, nanoparticles need to overcome a multitude of biological and physical barriers to reach targeted cell populations in the body. Second, once nanoparticles reach targeted cells, several cellular barriers need to be overcome before nanoparticles can interact with intracellular proteins and organelles. In addition to these hurdles, nanoparticles and/or their payloads need to accumulate in targeted cellular structures at sufficiently high concentrations to elicit the desired biological and/or therapeutic effect.

4.3. Exocytosis

In an effort to maintain cellular homeostasis, cells must undergo exocytosis. Exocytosis is characterized by the expelling of membrane-wrapped vesicles and its contents to the extracellular space. Exocytosis plays an important role in proper receptor functioning as endosomes often recycle endocytosed receptors and traffic them back to the cell periphery (Fig. 5 civ) [227]. Notably, this process is critical for receptors like the glucose transporter 4 and the neonatal Fc receptor [228,229]. On the other hand, lysosomes also play a role in exocytosis. In fact, lysosomal-mediated exocytosis is one of the most prominent forms for nanoparticle excretion from cells (Fig. 5g) [230]. Interestingly, direct translocation of nanoparticles from the cytoplasm across the cell plasma membrane and into the extracellular space is much less favored than vesicle-mediated exocytosis (Fig. 5g) [231].

Although nanoparticle internalization has been extensively studied with many different applications and modifications, the excretion of nanoparticles from cells is less defined. As with nanoparticle entry, nanoparticle exit also seems to be cell-dependent. Such a conclusion was drawn on internalization studies performed with 50-nm silica nanoparticles using three different human cell lines: (i) human lung carcinoma (H1299); (ii) human esophageal epithelia (NE083); and (iii) human bronchial epithelial (NL20) [231]. Studies using these three different cell lines revealed similarities between the cell lines during endocytosis of nanoparticles. However, the excretion processes were markedly different among these cell lines. The researchers reported that silica nanoparticles remained inside esophageal epithelia cells for a longer period than inside the lung carcinoma and bronchial epithelial cell lines. It was also noted that endo- and exocytosis processes occurred simultaneously.

Research has been devoted to reducing nanoparticle exocytosis rates to allow prolonged intracellular residency of nanoparticles for enhanced payload delivery. For example, Kim *et al.* harnessed strategies from supramolecular chemistry to delay the exocytosis of intracellular gold nanoparticles from MCF-7 breast cancer cell line [232]. Gold nanoparticles modified with quaternary amine head groups were internalized by MCF-7 cells. Then, subsequent *in situ* treatment with cucurbit [7] uril (CB [7]) formed a complex with the amine head groups which resulted in the assembly of aggregated gold nanoparticles within the cell. The large aggregated gold nanoparticle complexes remained sequestered in the endosomes. TEM and inductively-coupled plasma mass spectrometry (ICP-MS) analysis suggested that the aggregated structures entirely avoided exocytosis over the course of 24 hours without any observed cytotoxicity.

In another study, Yanes *et al* decreased the rates of exocytosis of mesoporous silica nanoparticles with the hope of promoting retention within cancer cells for enhanced payload delivery [233]. By inhibiting lysosomal-mediated exocytosis, enhanced *in vitro* cell killing was observed due to improved cellular retention of camptothecin-loaded nanoparticles. However, more research on nanoparticle exocytosis is needed to enable nanomedicine researchers to engineer nanoparticles with defined intracellular trafficking, pharmacokinetics, and exocytosis characteristics. Timely exit of nanoparticles from intracellular compartments is important for the design of nanomedicines to avoid the onset of adverse side effects.

Exocytosis is also a key component for immunity. Different immune cells like granulocytes, natural killer cells, and cytotoxic T-cells exocytose granules to affect defensive immune responses. In particular, stimulated cytotoxic lymphocytes release granules that contain perforin to form holes in the membranes of target cells to induce cell death [234]. Jones and coworkers leveraged the exocytosis of granules from cytotoxic lymphocytes to develop perforin-responsive nanoparticles [235]. First, drug-loaded lipid nanoparticles were loaded to the surfaces of cytotoxic T-lymphocytes. Upon antigen binding with the cytotoxic T lymphocyte, the secreted perforins degraded the lipid nanoparticles which resulted in released cargo as shown in Fig. 8. The group used HIV-specific cytotoxic lymphocytes loaded with lipid nanoparticles that had encapsulated IL-15Sa. The cytotoxic lymphocytes loaded with nanoparticles exhibited superior antiviral activity *in vivo* by lowered numbers of HIV-infected (Gag^+) $CD4^+$ T-cells when compared to empty lipid nanoparticles. This proof-of-concept study exploits the exquisite specificity of T-cells as well as their exocytotic behavior to elicit environmentally responsive nanoparticle immunotherapy delivery.

In this chapter, we provided a concise overview of the complex intracellular environment that nanoparticles may experience. Effective strategies to elicit biological and/or therapeutic responses often require that nanoparticles can overcome intracellular barriers efficiently and interact with desired intracellular targets. This requires efficient intracellular transport and trafficking of nanoparticles. As highlighted in this chapter, nanoparticles can be designed to respond and exploit endosomal/lysosomal environments for escape from these intracellular compartments. These escape mechanisms are important prerequisites for downstream transport to cytoplasmic destinations and intracellular organelles. In addition to cellular uptake of nanoparticles, exocytosis is an important intracellular process that needs to be considered when designing nanoparticles for intracellular applications. In the next chapter we focus on the kinetics of nanoparticle cellular uptake, and intracellular trafficking processes.

5. Kinetics of nanoparticle-cell interactions

As discussed in previous chapters, nanoparticle cellular uptake and intracellular transport depend on both nanoparticle physicochemical properties (*e.g.*, size, shape, composition, surface chemistry) and characteristics of the biological system, including cell type and function. While nanoparticle-cell interactions are prerequisites for effective application of nanomedicines *in vitro* and *in vivo*, the rates at which these interactions occur are complex and need to be investigated and understood

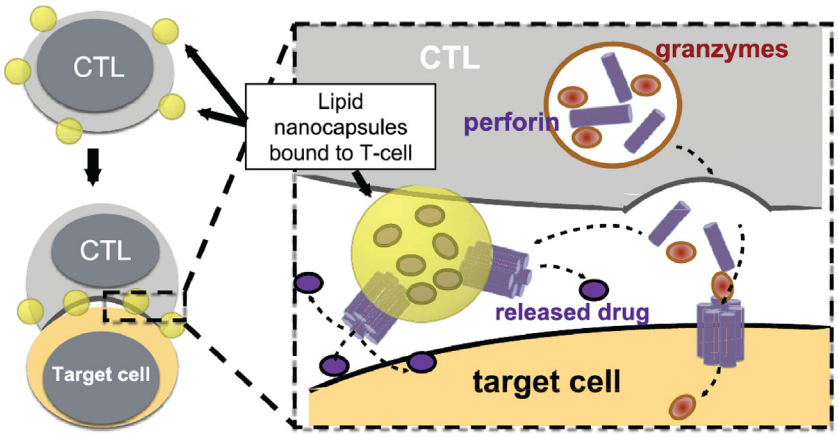

Fig. 8. Perforin-responsive lipid nanoparticles adhered to cytotoxic T-Lymphocytes. By relying on the unparalleled specificity of the T-cell receptors on cytotoxic lymphocytes to viral peptides on MHC-1 molecules, the researchers obtained antigen-specific release of drug payloads due to perforin exocytosis and subsequent nanoparticle/target cell membrane disruption. Abbreviations CTL: cytotoxic T-lymphocyte. Reproduced with permission from Ref [235].

(Fig. 10). This understanding is important as the kinetics of nanoparticle cellular entry, intracellular trafficking, degradation, payload release, and exocytosis determine pharmacokinetic and toxicological profiles as well as therapeutic efficacy. Such data on nanoparticle intracellular transport rates and pharmacokinetics will ultimately inform the engineering of more effective nanomedicines to facilitate clinical translation.

Upon *in vivo* exposure, nanoparticles interact with different parts of the body and may ultimately be processed by cells. To investigate the mechanisms involved in nanoparticle biodistribution and *in vivo* processing, Kolosnjaj-Tabi and coworkers tracked the one-year fate of iron oxide-coated gold nanoparticles in mice [236]. The nanoparticles were comprised of a 5-nm diameter gold core and coated with iron oxide to form a hybrid core-shell architecture with an average nanoparticle diameter of 16 nm. Nanoparticles were modified with one of two surface ligands: (i) amphiphilic polymers; or (ii) catechol-derived PEG ligands. Irrespective of the surface modification, nanoparticles were taken up by cells and detected in intracellular vesicles of liver Kupffer cells and splenic Ito cells even one year after intravenous administration. Authors reported that one year after nanoparticle administration the iron oxide shell of PEG-coated hybrid nanoparticles had been degraded and removed from both the liver and spleen, whereas the amphiphilic polymer-coated nanoparticles retained up to 10% of the initial iron oxide dose in these organs. This study demonstrated that nanoparticle composition and surface chemistry can determine *in vivo* distribution and degradation rates, which need to be considered when designing nanoparticles for biological applications.

Similar studies that assess the fate of nanoparticles *in vivo* can provide much-needed insight for the nanomedicine community about biodistribution, degradation, and elimination of nanoparticles introduced into biological systems [237,238]. Data from such studies may help addressing translational questions concerning nanoparticle dosing, administration frequency, rates of nanoparticle degradation in organs and cells, and their long-term toxicological potential.

In this chapter, we focus on the underlying nanoparticle kinetics for cellular uptake and intracellular transport that affect downstream biological and therapeutic responses of nanomedicines.

5.1. Kinetics of nanoparticle cellular uptake

Nanoparticles enter cells *via* multiple different pathways, including endocytosis and direct translocation into the cell's cytoplasm (Chapter 2). To elucidate the kinetics of these processes, Lunov and coworkers investigated cellular uptake rates of iron oxide nanoparticles by human macrophages in tissue culture [239]. The iron oxide nanoparticles were surface-coated with carboxydextran and exhibited diameters of 20 nm and 60 nm, respectively. Within 60 minutes after exposure, nanoparticles were observed in intracellular vesicles (likely endosomes) of macrophages as shown by TEM. Interestingly, macrophages internalized 60-nm nanoparticles 10x times more efficiently than 20-nm counterparts. This finding re-emphasizes the size-dependence of nanoparticle-cell interactions that we discussed in Chapter 3. The researchers used a quantitative bathophenanthroline disulfonic acid-based chromogenic assay to determine the number of internalized iron oxide nanoparticles using spectrophotometry. Based on these results, a mathematical model was developed to account for uptake parameters, such as the rate of nanoparticle uptake, mean time of uptake, number of nanoparticles that are inside the cells at the saturation point, and the connection between extracellular and intracellular nanoparticles. Eq. (1) describes the number of endocytosed nanoparticles as a function of time:

$$N(t) = N_s\left(1-e^{-t/T}\right) \tag{1}$$

where N(t) is the number of internalized nanoparticles at time *t*; N_S is the number of internalized nanoparticles at saturation as *t* approaches infinity; T is the characteristic time of nanoparticle uptake by a macrophage. Determination of the maximum nanoparticle uptake rate by macrophages is given by Eq. ((2):

$$\frac{dN(0)}{dt} = \frac{N_s}{T} \tag{2}$$

The two equations outlined in this study offer predictive power to researchers looking to better control nanoparticle-cell interactions *in vitro*.

To shed more light on how nanoparticle uptake is impacted by factors like the formation of a protein corona, Lesniak and coworkers fluorescently labeled 40-nm carboxylated polystyrene nanoparticles and calculated their entry in human cells using flow cytometry [240]. The researchers concluded that the rates of nanoparticle cellular uptake, such as endocytosis, are based on two processes; firstly, nanoparticles cling to a membrane where they encounter surface proteins and lipids; following the short adsorption (Fig. 10, rate constant 'k_{on}') onto the membrane's surface, nanoparticles are then internalized (Fig. 10, rate constant 'k_{end}'). The two-step kinetic operation of nanoparticle-cell uptake was described by authors using Eq. (3):

$$J(c_0) = N_{m,\,max}k_{m1}/(1 + (k_{m0} + k_{m1})/k_{0m}c_0) \tag{3}$$

where J is the nanoparticle uptake rate of an initial concentration (c0) of extracellular nanoparticles; nm,max is the maximum possible number of nanoparticles absorbed onto cell plasma membrane; km1 is the

internalization rate; while k_{0m} and k_{m0} are the rates of adsorption and desorption from the plasma membrane, respectively. The researchers highlighted the significant impact that the presence of a nanoparticle protein corona has on nanoparticle cell uptake. In comparison to bare nanoparticles, the protein corona reduces adhesion of nanoparticles to the cell membrane. However, certain biomolecules within the protein corona may induce specific recognition by the cell plasma membrane to facilitate regulated nanoparticle cellular uptake with altered internalization kinetics. Hence, interactions and adhesion between nanoparticles and cell plasma membranes are key determinants of nanoparticle cellular uptake kinetics.

In another study, Blechinger and coworkers looked at the kinetics differences in cellular uptake of fluorescently-labeled silica nanoparticles between HeLa cancer cells and non-cancerous human vascular endothelial cells in tissue culture [241]. After 4 hours of nanoparticle exposure, the non-cancerous endothelial cells had 10x times higher concentrations of nanoparticles than the HeLa cells. The endothelial cells were relying on clathrin-mediated endocytosis, whereas the uptake route for the HeLa cells was not clear. One possible explanation for these results is the differences in metabolism and rates of proliferation among the two cell types. Following 10- and 24-hour incubation times with the silica nanoparticles, the HeLa cancer cells had a drastic increase of internalized nanoparticles which was calculated to be up to 2x times more than the number found in the human vascular endothelial cells. These results showcase the nonlinearity of nanoparticle uptake rates as well as the differences in uptake rates between different cell types.

Although endocytosis is a prominent pathway for nanoparticle cellular uptake, multiple pathways for cellular entry are available and may occur in tandem (Chapter 2). To that end, Lin and Alexander-Katz detailed how nanoparticles modified with CPPs cross the membrane directly and also undergo endocytosis [242]. Using coarse-grain molecular dynamics simulations, the authors determined that the negatively charged cellular plasma membrane experiences local shifts as more and more cationic particles diffuse towards the surface. At a critical concentration of cationic nanoparticles, pores begin to form in the cell plasma membrane within a matter of 40 nanoseconds (ns). Nearly 20 ns after, the holes were simulated to assume their initial sealed state. However, the overall electrical potential of the membrane surface is depleted after a certain number of nanoparticles rapidly have traversed the membrane. This disruption of the transmembrane potential prohibits nanoparticles to directly translocate through the cell plasma membrane. Consequently, the nanoparticles then rely on endocytosis for cellular uptake. Eventually the transmembrane potential is restored with the efflux of Ca^{2+} ions. The rate of restoring the transmembrane potential therefore modulates the rate of direct membrane translocation for cationic nanoparticles. This kinetic study by Lin and Alexander-Katz reemphasizes the impact of how nanoparticle surface charge and subsequent interactions with cellular plasma membranes can be exploited to efficiently deliver nanoparticles to cells and intracellular targets.

In summary, these studies underscore the details of how nanoparticle size, surface charge the cell type, and the cells' transmembrane potential affect the rates of nanoparticle internalization. The incorporation of these parameters can facilitate the design of nanoparticles with defined cellular uptake rates which could be used to control toxicological and pharmacokinetic profiles of nanomedicines.

5.2. Intracellular nanoparticle kinetics

Once inside cells, nanoparticles are exposed to a plethora of potential intracellular transport pathways that are often dictated by intracellular vesicles, such as endosomes, and/or nanoparticle surface chemistry (Figs. 5 and 6). As we discussed in Chapter 4, acidification of endosomes is a hallmark of intracellular vesicle trafficking (Fig. 5 ci). To track and quantify the acidification kinetics of endocytotic organelles, Wang *et al.* developed a novel class of "nanobuffers" [243]. These "nanobuffers" were made from ionizable amphiphilic block copolymers that self-assembled into micelles. At pH levels below the pK_a of the copolymers, the micelles dissociated. Based on these pH-dependent micelle transitions, the researchers were able to monitor pH changes by incorporating fluorophores into the micelles. BODIPY fluorescence signals were achieved at specific pH ranges which enabled the real-time measurements of endo/lysosomal pH levels in HeLa cancer cells exposed to micellar "nanobuffers". To calculate the rate of acidification, the researchers determined that ~64,000 amino groups from the polymer are imbedded in the polymeric micelle. The researchers also determined an average of 200 nanoparticles were sequestered in endosomes/lysosomes, leading to an acidification rate of ~140-190 protons per second. Such nanoscale polymer-based probes provide valuable information concerning time-resolved intracellular transport kinetics of vesicles and nanoparticles. Understanding of downstream intracellular events can provide guiding principles to researchers for rational engineering of more effective nanomedicines.

In a different study, Liu and coworkers employed 50-nm gold nanoparticles modified with surface-bound dsDNA to describe the time-dependent intracellular evolution of nanoparticles within the endo/lysosomal pathway [244]. By relying on fluorescence and surface plasmon resonance, researchers were able to observe gold nanoparticles inside early endosomes of HeLa cancer cells *in vitro*. Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) revealed that nanoparticle cell uptake was time-dependent and plateaued around 8 hours. Total internal reflection fluorescence microscopy revealed that within 2 hours, single nanoparticles were observed in early endosomes. However, once endosomes began to age (4–12 hours post incubation) the gold nanoparticles began to cluster within late endosomes and lysosomes. Dark-field microscopy was then used to confirm that the clustering states of the gold nanoparticles impacted the intracellular transport of the nanoparticles in real time. Single nanoparticles trapped in early endosomes exhibited high motility, whereas large gold nanoparticle clusters found in late endosomes/lysosomes near the perinuclear region exhibited low motility. This study details the steps of intracellular nanoparticle transformations within late endosomes/lysosomes and implies that changes in nanoparticle physicochemical properties and aggregation states can occur during intracellular trafficking. Moving forward, quantitative studies may be valuable that investigate intracellular alterations of nanoparticle properties, as these changes may affect biological responses and therapeutic efficacy of nanomedicines.

We discussed in Chapter 4 that nanoparticle surface modifications can facilitate endosomal escape and intracellular organelle localization. To examine this phenomenon more deeply, Krpetić *et al.* modified 14-nm gold nanoparticles with CPP sequences to study how these nanoparticles can overcome intracellular barriers [245]. Using TEM, the researchers identified the presence of gold nanoparticles within the cytoplasm and organelles of HeLa cancer cells after two hours of nanoparticle exposure (Fig. 9a). Within 10 hours of exposure, gold nanoparticles were reported to be dispersed in the cytoplasm and in organelles like mitochondria and the nucleus (Fig. 9b). Later TEM images after 24 hours showed these unbound nanoparticles were entrapped in intracellular vesicles. Interestingly, the entrapped nanoparticles were reported to escape the vesicles *via* membrane rupture and direct translocation (Figs. 9c and 9d). In addition to vesicle escape, the nanoparticles were seen clustered in large numbers in vesicles 24 and 48 hours later (Figs. 9e and 9f). A nanoparticle system with the ability to traverse multiple intracellular membranes as described by Krpetić and coworkers provides a potential platform for applications in gene and drug delivery to reach intercellular and cytoplasmic targets more efficiently.

To examine nanoparticle cellular entry and exit mechanisms more closely, Jiang *et al.* incubated HeLa cancer cells with zwitterionic quantum dot nanoparticles (~8 nm in diameter). The researchers observed that nanoparticles entered cells through clathrin-mediated endocytosis

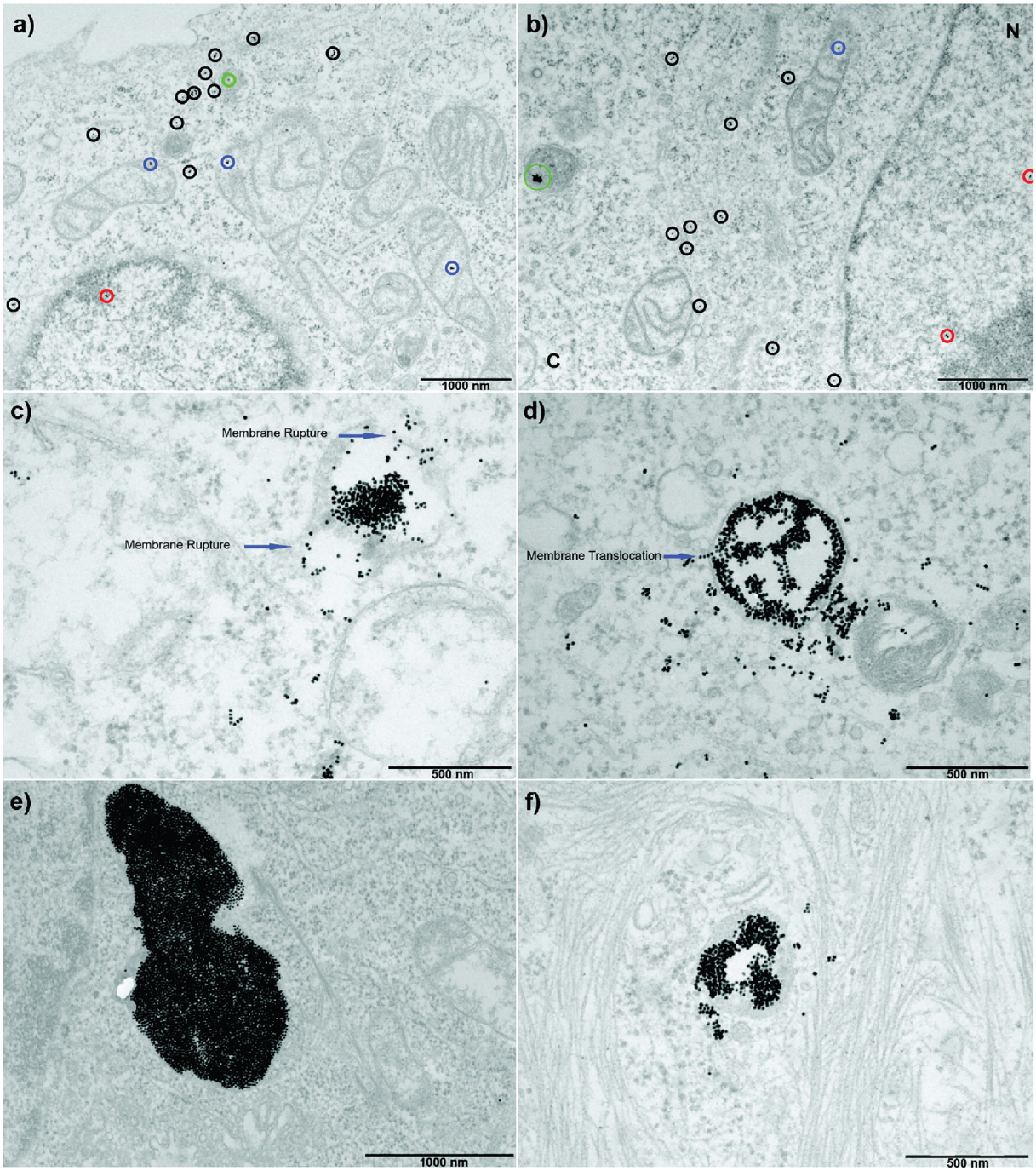

Fig. 9. Time-dependent intracellular trafficking of TAT-modified gold nanoparticles in HeLa cells. (a) After two hours of incubation, TEM reveals nanoparticles largely in the cytoplasm (black circles). The nanoparticles are also seen in mitochondria (blue circles), nucleus (red circle), and in endosomal-like vesicles (green circle). (b) After ten hours, a similar distribution as shown in (a) is observed. (c, d) Twenty-four hours post incubation, nanoparticles are seen to be releasing from intracellular vesicles through both membrane rupture and membrane translocation (blue arrows). (e, f) Additionally, 24 and 48 hours post incubation, nanoparticles are densely packed inside intracellular vesicles through unknown mechanisms. Adapted with permission from Ref. [245] Copyright 2011 American Chemical Society.

[246]. Nanoparticles clustered around the cell membrane but were not enveloped into HeLa cells until a certain density of nanoparticles had attached to the membrane. These findings demonstrate a dose/concentration-dependent nanoparticle cellular uptake that is a characteristic of nanoparticle cell uptake kinetics parameters outlined in Eq. (3). Once the critical nanoparticle concentration at the cell plasma membrane was achieved, the quantum dot nanoparticles were seen inside endosomes. These intracellular vesicles were then transported along microtubules to other regions of the cell with trajectory lengths of >3 μm. Surprisingly, after 2 hours of nanoparticle exposure, more than half of the internalized quantum dots were exocytosed from the HeLa cells leading to a calculated intracellular half-life of the quantum dots of only 21 minutes. Such a relatively short intracellular half-life could be insufficient for nanoparticles that rely on late endosomes/lysosomes to payload release and delivery. Furthermore, this short nanoparticle intracellular residence time could prevent nanoparticles from acting as long-term diagnostic tools. Therefore, a balance must be met for diseased cells to have enhanced nanoparticle uptake while also maintaining control over rates of nanoparticle exocytosis and intracellular processing.

In summary, the intracellular characteristics of nanoparticles are in a state of constant flux and change dynamically. As nanoparticle physicochemical properties and aggregation states change within cells, their drug and imaging payloads are often subject to alterations as well. These intercellular dynamics can inactivate genetic material payloads, such as siRNA and DNA, or affect small molecule functionality and efficacy. As to how this shifting environment impacts nanoparticle payload delivery rates is still an active area of research. In the next section we briefly highlight efforts into determining the intracellular time-dependent delivery of payloads from nanoparticles.

5.3. Intracellular nanoparticle pharmacokinetics

Although nanoparticles can be designed to release their payloads inside cells, the mechanisms of how these processes function are not fully understood. To address this gap in the current understanding of intracellular nanoparticle kinetics, quantitative studies are needed that assess intracellular pharmacokinetics, pharmacodynamics, and intracellular nanoparticle trafficking, including parameters such as nanoparticle dosage, endosomal escape, payload release rate, metabolism, and excretion. To that end, Soininen and colleagues used liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to detail the intracellular doxorubicin release profiles from liposomes *in vitro* [247]. By quantifying the concentrations of doxorubicin at different time points with LC-MS/MS and measuring cell death as a function of doxorubicin accumulation within the nucleus. The following equations (Eqs. (4)–(6)) were derived using a two-compartment transit compartment model (Fig. 11) to model intracellular pharmacokinetics-pharmacodynamics (PK/PD) relationships of PEGylated liposomal doxorubicin nanoparticles (CAELYX).

$$K = \frac{K_{max}(C_{nucl} - C_{thr})}{(EC_{50} - C_{thr}) + (C_{nucl} - C_{thr})} \tag{4}$$

K is the initial cell kill rate constant, K_{max} is the maximum cell kill rate constant, C_{nucl} is the concentration of doxorubicin inside the nucleus, C_{thr} is the threshold nuclear concentration of doxorubicin to elicit a response where ($C_{nucl} - C_{thr} \geq 0$), EC_{50} is the nuclear concentration of doxorubicin to elicit 50% of K_{max}.

$$\frac{dk_1}{dt} = \frac{1}{t}(K - K_1), \frac{dk_2}{dt} = \frac{1}{t}(K - K_2) \tag{5}$$

K_1 and K_2 are the delayed and flattened cell kill rate constants in the first and second compartments, respectively, t is the delay time due to transit between the compartments.

$$\frac{dV}{dt} = -K_2 V \tag{6}$$

where dV/dt is cell viability (V) as a function of time which is based on the rate of doxorubicin accumulation in the second compartment (*i.e.*, cell nucleus).

Soininen and coworkers reported that nuclear accumulation of doxorubicin was dependent on the cell type and on the intracellular concentration of doxorubicin. Liposomal doxorubicin had a greater impact on decreasing cell viability than free doxorubicin indicating that encapsulated doxorubicin does not lose its biological functionality. This study provided a PK/PD model for liposomal doxorubicin to describe time-resolved drug concentration-therapeutic response relationships.

Li *et al.* incorporated paclitaxel into polymeric PLGA nanoparticles to quantitively describe the drug release and encapsulation profile *in vitro* using high-performance liquid chromatography (HPLC) [248]. This pharmacokinetic analysis used 11 parameters related to specific rate constants to determine the intracellular release of paclitaxel from PLGA nanoparticles. Although this *in vitro* system does not fully recapitulate the *in vivo* situation, these data can be integrated to pharmacokinetic models to computationally model and inform dosing regimens for potential nanomedicine therapies.

Thorough understanding of payload kinetics is particularly important for nanoparticle-mediated gene editing and gene regulation approaches. Mechanistic studies on the delivery of DNA/RNA-loaded nanoparticles offer insights into intracellular delivery efficiencies and potential intracellular barriers. For instance, in a study by Wittrup *et al*, cationic lipid nanoparticles formulated with siRNA were shown to only release their payload within ~10 minutes post-endocytosis *in vitro* in HeLa cells [249]. Additional analysis showed siRNA release occurred in maturing endosomes and the cytoplasmic delivery efficiency was calculated to only ~3.5% of the total siRNA internalized. This study highlights the challenges with cytoplasmic delivery of nucleic acids-based nanoparticle payloads.

Further examination into the delivery efficiency of nanoparticles encapsulating siRNA was conducted by Gilleron *et al.* [250]. They reported that after 6 hours of incubation, lipid nanoparticles encapsulating siRNA sequences were seen to be clustered in early and late endosomes as well as in lysosomes of HeLa cells. The researchers further demonstrated that escape from these intracellular vesicles is a critical rate-limiting step in siRNA interventions. To better understand the intracellular trafficking patterns, the researchers doped their lipid nanoparticles decorated with siRNA with a gold nanoparticle core to enable visualization of intracellular distribution *via* electron microscopy. The gold-containing lipid nanoparticles were observed in intracellular vesicles at 1.5 hours. Six hours after exposure, the nanoparticles were trapped in lysosomes. Since siRNA must maintain its structural integrity and enter the cytoplasm to elicit the desired therapeutic/biological response, these observed nanoparticle trafficking behaviors represent significant barriers that may limit the efficacy of siRNA-based therapeutic strategies. *In vivo* biodistribution analysis showed that the gold-containing lipid nanoparticles were trapped within endosomes and lysosomes of Kupffer cells and hepatic-resident macrophages 30 minutes post injection. This rapid sequestration of nanoparticles by the liver poses a challenge to efficient nanoparticle delivery to diseased cells in the body.

Likewise, Wu and coworkers reported endosomal entrapment of spherical nucleic acids (SNAs) with gold nanoparticle cores in tissue culture [251]. Post-endocytosis of SNAs, the gold nanoparticles resided in late endosomes over the course of 24 hours. The spherical nucleic acid shell had been degraded within 16 hours and excreted from the cells while the gold core persisted in late endosomes. These time points impose valuable kinetic parameters that are crucial for effective delivery of genetic material before intracellular degradation of nucleic acids. Given these challenges, new approaches in nucleic acid encapsulation and delivery have been explored. For example, unique polymeric and lipid-based nanoparticles that exhibit better protection of the nucleic acid payload as well as stimuli-responsive nanoparticles can enhance intracellular delivery of gene disrupting and gene regulating payloads [252,253].

In summary, efficient intracellular delivery of nanoparticles and nanoparticle payloads is challenging with multiple different barriers that nanoparticles need to overcome to effectively interact with intracellular targets. Researchers need to consider additional intracellular challenges that are related to nanoparticle trafficking and pharmacokinetics, including rates of nanoparticle endocytosis, endosomal escape, intracellular transport, metabolism/degradation, and exocytosis (Fig. 10). We explored these multiparametric intracellular processes which represent active areas of current bionanotechnology research. Further studies of intracellular nanoparticle PK/PD using *in vivo* systems may provide findings that go beyond tissue culture-based *in vitro* studies to better assess the translational potential of nanomedicines.

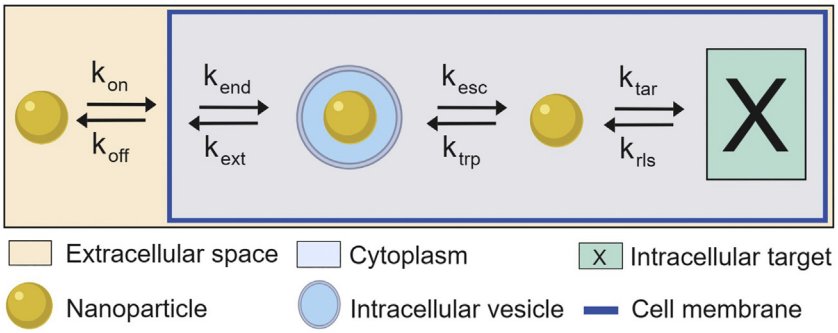

Fig. 10. Compartmental overview diagram depicting major nanoparticle cellular interactions and intracellular transport pathways. Nanoparticles in the extracellular space interact with the cell plasma membrane (k_{on}, k_{off}) to enter cells (k_{end}). After successful cellular entry, nanoparticles then need to escape (k_{esc}) intracellular vesicles (*e.g.*, endosomes) to engage with intracellular targets (k_{tar}). Cytoplasmic nanoparticles can be trapped back in vesicular structures (k_{trp}) or released from intracellular targets (k_{rls}). The excretion of nanoparticles to the extracellular space is defined by k_{ext}. 'k' denotes rate constants for each of these individual steps.

6. Future directions and conclusions

Nanoparticles can be engineered from inorganic and organic materials with unique physical, chemical, and biological properties for applications in medicine. Once administered into the body, nanoparticles interact with different tissues and cells. While specific and efficient delivery of nanoparticles to diseased tissue sites and cells in the body is challenging [17,18], nanomedicine offers the potential to transform diagnostic and therapeutic strategies. However, more quantitative studies that explore and assess essential mechanisms of nano-bio interactions in great detail are needed to provide solutions for overcoming biological and physical barriers that currently limit the clinical translation of nanomedicines [4].

There is a large body of preclinical and clinical work available in the literature that describes applications of organic and inorganic nanoparticles for various medical applications including: vaccines [254–260], anti-microbials [261–265], medical imaging [266–270] and diagnosis [271–274], as well as various approaches in cancer treatment [275–280] and chronic kidney disease [281,282]. Recent studies have reported the use of stimuli responsive nanoparticles that can be engineered for applications in cancer management to overcome biological barriers [283–287]. Other work has explored the application of nanoparticles for immunoengineering and immunotherapy strategies [288–291]. One intriguing concept is the use of "albumin hitchhiking" for immunotherapy applications [292,293]. Liu *et al.* uncovered that amphiphilic compounds with lipophilic domains can adhere to circulating albumin which is then efficiently transported to lymph nodes [294]. With thorough optimization, the amphiphilic molecules resulted in enhanced lymph node accumulation. Upon conjugation of antigens and adjuvants, the group noticed strong T-cell priming, anti-tumor efficacy, and reduced systemic toxicity. The potential of this lymph node targeting concept was further highlighted by the elimination of large established tumors in preclinical mouse models *via* combination immunotherapies [295]. Such promising results are examples of the growing field of nano-immunoengineering. As this field grows, more research devoted to elucidating and fine tuning the interactions between nanoparticles and the cellular/tissue components of the immune system will offer the potential for enhanced clinical translation of novel nanoparticle-based immunotherapies.

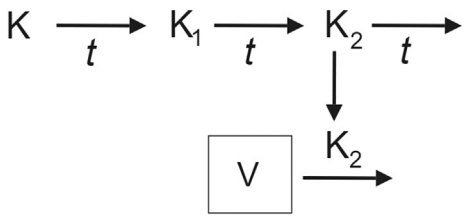

Fig. 11. Schematic overview of pharmacokinetic-pharmacodynamic transit compartment model. 'K' is the initial cell kill rate constant, 'K_1' and 'K_2' are the cell kill rate constants in the transit compartments, t is the mean transit time, and V is the cell viability. The initial cell kill rate constant is a nonlinear function of nuclear doxorubicin concentration. Adapted with permission from Ref. [247] Copyright (2016) American Chemical Society.

Our article offers an overview of fundamental interactions between nanoparticles and cells, including immune cells. Such understanding is important for researchers to design nanoparticles for enhanced cellular uptake and intracellular transport with defined pharmacokinetics and therapeutic/biological performance. Since interactions between nanoparticles and cells are complex and multiparametric, including parameters such as nanoparticle size, shape, surface charge, elasticity, and biological characteristics of cells, better fundamental understanding of these essential nano-bio interactions is required. With the development of new analytical techniques in recent years, the transport of *in vivo* administered nanoparticles can be assessed with spatiotemporal information that can ultimately guide the engineering of more effective nanomedicines. These approaches include single-cell elemental analysis methods, such as single-cell inductively-coupled plasma mass spectrometry (SC-ICP-MS) [296], and mass cytometry [297], as well as new methods for three-dimensional optical microscopy of intact organs and tissues with subcellular resolution [298–303]. These new analytical methods provide researchers with powerful tools to study the fate of administered nanomedicines in the body with cellular and subcellular precision.

To accelerate the clinical translation of nanomedicines, improved understanding of nanoparticle delivery barriers is needed. Systematic quantitative studies that elucidate the mechanisms of interactions between nanoparticles and biological systems may provide guiding principles for the design of more effective nanomedicines with the ultimate goal to overcome delivery barriers and to better control the transport of nanoparticles in the body. These studies need to take into account the complexity of nano-bio interactions that occur at various different levels including organ, tissue, cellular, subcellular, and biomolecular levels. We believe that a holistic understanding of nano-bio interactions will accelerate clinical translation of nanomedicines, including nano-immunotherapies.

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