Journal of Controlled Release 190 (2014) 485–499

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Insight into nanoparticle cellular uptake and intracellular targeting ☆

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ARTICLE INFO

Article history:
Received 13 May 2014
Accepted 21 June 2014
Available online 28 June 2014

Keywords:
Nanomedicine
Nanoparticle cellular uptake
Non-targeted and targeted nanoparticles
Intracellular distribution
Subcellular targeting

ABSTRACT

Collaborative efforts from the fields of biology, materials science, and engineering are leading to exciting progress in the development of nanomedicines. Since the targets of many therapeutic agents are localized in subcellular compartments, modulation of nanoparticle–cell interactions for efficient cellular uptake through the plasma membrane and the development of nanomedicines for precise delivery to subcellular compartments remain formidable challenges. Cellular internalization routes determine the post-internalization fate and intracellular localization of nanoparticles. This review highlights the cellular uptake routes most relevant to the field of non-targeted nanomedicine and presents an account of ligand-targeted nanoparticles for receptor-mediated cellular internalization as a strategy for modulating the cellular uptake of nanoparticles. Ligand-targeted nanoparticles have been the main impetus behind the progress of nanomedicines towards the clinic. This strategy has already resulted in remarkable progress towards effective oral delivery of nanomedicines that can overcome the intestinal epithelial barrier. A detailed overview of the recent developments in subcellular targeting as a novel platform for next-generation organelle-specific nanomedicines is also provided. Each section of the review includes prospects, potential, and concrete expectations from the field of targeted nanomedicines and strategies to meet those expectations.

1. Introduction

Multidisciplinary and integrative research efforts in the field of nanomedicine have led to the development of a variety of nanoparticle-based carrier systems suitable for site-specific delivery of diagnostic and therapeutic agents [1]. The original foundation for the recent dramatic progress in the use of nanomaterials for biomedical applications is considered to be the famous 1960 lecture of R. Feyman, "There is plenty of room at the bottom" [2]. However, the work of Paul Ehrlich, who coined the visionary term "magic bullets" to describe cell-specific diagnostics and cell-targeted therapies, is also of seminal importance [3,4]. The field of nanomedicine has established its capability to overcome the low solubility, non-specific cytotoxicity, poor bioavailability, and suboptimal pharmacokinetics and pharmacodynamics associated with the cytotoxic agents employed in cancer chemotherapy. With some nanomedicines already making their way into the clinic, liposomes, polymeric nanoparticles, dendrimers, and gold nanoparticles have demonstrated remarkable potential as carrier systems [5–8]. On one hand, the entire field of nanomedicines has been greatly expanded by the development of a wide range of nanomaterials with a high degree of control over their physical (e.g., size, surface charge, shape, mechanical strength) and chemical attributes. At the same time, a better understanding of the physiopathological nature of different diseases and insight into the interaction of nanomaterials with biological systems at various levels (i.e., systemic, organ, tissue, and cell) are of paramount importance for further progress towards bench-to-bedside translation. The recent strides forward in nanomedicines stem from some key multidisciplinary efforts. The non-fouling nature of hydrophilic materials such as polyethylene glycol (PEG) and polycarboxybetaine (PCB) [9,10] against biological materials, and recognition of the enhanced permeability and retention (EPR) effect are two such examples [11]. The development of hydrophilic polymer functionalization at the surface of nanoparticles imparts a stealth character against the immune system and enhances their systemic circulation [12]. The groundbreaking discovery of the EPR effect [13,14], which stems from the abnormal and leaky microvasculature common to tumors, has laid the foundation for the first generation of passively targeted nanomedicines that preferentially accumulate in tumor tissue [15,16]. While the EPR effect has also been observed during inflammation caused by other diseases, in that context this review is mainly focusing on the EPR effect in tumor tissues. The combination of long systemic circulation made possible by hydrophilic polymers and the EPR effect results in the accumulation of nanoparticle-based carrier systems in the tumor tissue followed by the release of therapeutic agent, either in proximity to diseased tissue or inside the cells after internalization. The EPR effect results from many complex biological processes including differences in

☆ This review is part of the 30th Anniversary Issue of the Journal of Controlled Release.

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http://dx.doi.org/10.1016/j.jconrel.2014.06.038
0168-3659/

cancer genetics as reviewed in ref. [11], consequently the therapeutic outcomes based on exploiting the EPR effect can be inconsistent due in part to the heterogeneity of tumor tissue. Recently, exploitation of the specific affinity of receptors to certain ligand molecules has led to the second generation of nanomedicines, which are preferentially targeted to particular organs, tissues, or cells. The ligands, with specific affinity towards a particular receptor or molecule differentially expressed at the target site, are displayed on the surface of nanocarriers, resulting in the preferential accumulation and uptake at the site of action [1,17]. Although some concerns have been raised about poor systemic circulation, enhanced clearance by the mononuclear phagocyte system, and limited tissue penetration, the new paradigm of ligand-conjugated actively targeted nanocarriers has been shown to improve the cellular uptake and efficacy of their payload when compared to their passively targeted counterparts [18,19]. The enhanced cellular uptake of nanoparticles at the disease site is of paramount importance, because targets for many theranostic agents against several disorders (including cancer) are localized in the subcellular compartments [20]. This fact not only highlights the importance of a better understanding of cellular uptake mechanisms but has also fueled recent research into the development of nanocarriers capable of subcellular- and organelle-level targeting, referred to as the third generation of nanomedicines [21]. After giving an account of the endocytic pathways relevant to non-targeted and ligand-conjugated targeted nanoparticles, we provide a comprehensive review of recent developments and outline future strategies in designing nanomedicines capable of efficient intracellular trafficking and subcellular targeting.

2. Endocytic routes and non-ligand targeted nanomedicines

Precise release of drugs in specific organs, tissues, and cells [22] has been the primary focus of nanoparticle-based therapeutic strategies. However, drug-loaded nanoparticles must overcome a number of transport barriers to reach their target [23]. Particularly for intracellular targeting, efficient translocation of nanoparticles across the plasma membrane barrier is a prerequisite. The plasma membrane is highly complex and provides an independent environment necessary to develop the normal function of different types of cells. This membrane also plays a critical role in cellular adhesion, communication, and division, and endocytosis is crucial to the regulation of these functions. Endocytosis involves the generation of new intracellular membrane-enclosed vesicles from the plasma membrane with a concomitant internalization of lipids, proteins, and extracellular fluid (Fig. 1). The opposite phenomenon, exocytosis, is the fusion of inner vesicles with the plasma membrane to transport molecules either to plasma membrane or to extracellular space [24]. Endocytic and exocytic trafficking are highly dynamic and well regulated, and it has been estimated that cells can internalize up to five times their volume and membrane area in one hour [25]. Phagocytosis and pinocytosis are the two main endocytic pathways employed by cells. Phagocytosis is mainly used by dendritic cells, neutrophils, and macrophages [26]. Pinocytosis occurs in all types of cells and can be further subdivided into clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin/caveolae-independent endocytosis, and macropinocytosis. Because efficient uptake of nanoparticles is central to effective intracellular drug delivery, we believe that a deeper understanding of the biological pathways for cellular internalization of nutrients and solutes can facilitate the development of nanoparticles with precise intracellular targeting and enhanced therapeutic outcomes.

2.1. Phagocytosis

Phagocytosis is an endocytic process exhibited by several types of cells, including epithelial cells, fibroblasts, immune cells, specific phagocytic cells (monocyte, macrophages, and neutrophils), cells that generate inflammatory mediators (basophils, eosinophils, and mast cells), and natural killer cells [26]. In mammalian organisms, phagocytosis is used to engulf disabled particles, senescent cells, and infectious

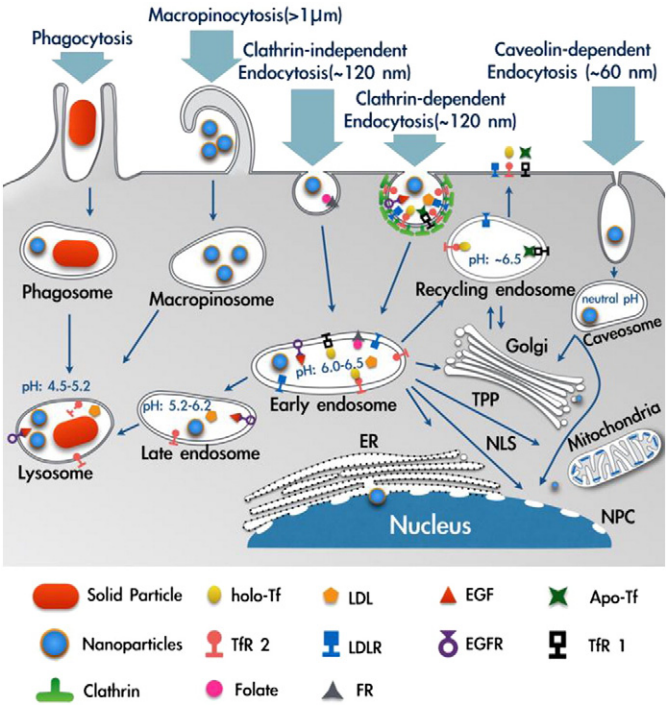


Fig. 1. Illustration of internalization pathways discussed in this article (phagocytosis, macropinocytosis, clathrin-dependent endocytosis, clathrin-independent endocytosis, and caveolae-dependent endocytosis). The fate of internalized cargo and localization to subcellular compartments are also depicted. ER: endoplasmic reticulum, NLS: nuclear localization signal, NPC: nuclear pore complex, TPP: triphenylphosphonium cation. Adapted and reproduced with permission from [90,92].

microorganisms (bacteria and viruses) as part of both the innate and adaptive immune response [27]. One of the main characteristics of this unique form of endocytosis is the large size of the endocytosed vesicles (>250 nm) known as phagosomes [28]. Phagocytosis can be triggered either through the interaction of cell-surface receptors with particular ligands presented by the foreign agent or through the interaction of specific cell-surface receptors with soluble factors that recognize the foreign agent and facilitate phagocytosis (opsonization). The soluble factors involved in opsonization include proteins of the complement system, antibodies, acetylcholine, laminin, fibronectin, C-reactive protein, and type-I collagen [29]. The most important receptors that participate in phagocytosis are the Fc receptor family for IgG (FcγRI, FcγRIIA, and FcγRIIA), the complement receptors (CR1, CR3, and CR4), and α5β1 integrin [30]. A great deal of scientific effort has been focused on controlling nanoparticle internalization via phagocytosis. The cellular internalization of nanoparticles via phagocytosis in macrophages involves attractive forces (i.e., van der Waals, electrostatic, ionic, hydrophobic/hydrophilic) between the cells and nanoparticle surfaces. In addition, the phagocytosis of nanoparticles can also be triggered by the receptor-mediated recognition of opsonins adsorbed on the surface of nanoparticles. Mitragotri and coworkers [31,32] discovered that the particle geometry can help in modulating their cellular internalization via phagocytosis. Different local particle shapes at the point of cell attachment generate different angles between the membrane and particle. This contact angle has a significant effect on the ability of macrophages to internalize particles via actin-driven movement of the macrophage membrane. Mitragotri et al. examined six different shapes of nanoparticles: spheres (radius 1.0–12.5 μm), oblate ellipsoids (major axis 4 μm, aspect ratio 4), prolate ellipsoids (major axis 2–6 μm, aspect ratio 1.3–3), elliptical disks (major axis 3–14 μm, aspect ratio 2–4, thickness 400–1000 nm), rectangular disks (major axis 4–8 μm, aspect ratio 1.5–4.5), and UFOs (sphere radius 1.5 μm, ring radius 4 μm). The authors demonstrated that elongated particles with higher aspect ratios are less prone to phagocytosis. Geng et al. [33] reported a similar finding.

Interestingly, a higher aspect ratio has also recently been associated with preferential localization into endosomes and lysosomes [34]. Thus, care should be taken when attempting to exploit the shape of particles for modulating phagocytosis and intracellular targeting simultaneously.

2.2. Pinocytosis

2.2.1. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the most studied process for trafficking of materials into eukaryotic cells. CME is a complex pathway that involves intercellular signaling, membrane recycling, and uptake of nutrients [35]. Vesicle formation starts with the participation of extensive protein machinery to induce a curvature in the membrane (e.g., epsin [36], amphiphysin [BAR domain, Bin/amphiphysin/Rvs] [37], endophilin [38], and the FCHo2 F-BAR domain [39]), adaptor protein complexes (e.g., the AP-2 heterotetrameric complex [a-b2-m2-s2] [40], AP180 [41]), and clathrin assembly lymphoid myeloid leukemia [CALM] protein [42]), which are essential for the formation of the spherical clathrin-coated pit [43]. The release of a vesicle from the plasma membrane occurs by the activity of GTPase dynamin, a protein that is assembled as a ring around the neck of a newly formed invagination [44,45]. When the coated vesicles are released, the clathrin pit is disassembled by the action of auxilin and heat shock cognate 70 (HSC70)-dependent proteins [46]. After internalization through CME, the uncoated vesicles are either guided to early endosomes or recycled to the plasma membrane surface. The vesicles can also be targeted to more mature endosomes and later to compartments such as lysosomes and multivesicular bodies. The majority of receptor-mediated cellular uptake of nanoparticles occurs through CME. Particular examples are provided below in the sections dealing with ligand-targeted nanoparticles. In the case of non-targeted nanoparticles, the uptake route depends on their physical attributes including particle size, shape and surface charge, and also on the type of cell line. Cationic nanoparticles ~100 nm in size derived from polylactide-*co*-polyethylene glycol (PLA-PEG) have been found to internalize exclusively via CME [47]. Poly(L-lysine), which is a cationic polymer functionalized at the surface of poly(lactide-*co*-glycolide) (PLGA) nanoparticles, has also been found to significantly enhance cellular uptake via CME [48]. Another study reported that mesoporous silica nanoparticles (~110 nm) labeled with fluorescein isothiocyanate were efficiently internalized into human mesenchymal stem cells (hMSCs) and adipocytes (3T3-L1) predominantly via CME [49]. Although the underlying mechanisms that mediate the internalization of nontargeted nanoparticles are not fully understood, it seems that the high rate of cellular internalization via CME under normal conditions concluding the main route of internalization for nanoparticles of sizes ~100 nm. In particular for positively charged nanoparticles, the predominant uptake through CME might also stem from the electrostatic interaction with negatively charged cell surface. To the best of our knowledge, there is no systematic study of nanoparticles ~100 nm in size investigating the effect of shape on cellular internalization route; all the relevant literature deals with spherical nanoparticles.

2.2.2. Clathrin-independent endocytosis

The clathrin-independent endocytosis (CIE) pathway was initially described as a mode of entry for a number of bacterial toxins and cell-surface proteins, and recently was proposed to participate in plasma membrane repair, cellular polarization, cellular spreading, and modulation of intercellular signaling [50]. CIE does not require the presence of coat proteins for vesicle formation and internalization; however, the actin and actin-associated proteins are important players for vesicle formation during CIE [51]. CIE involves different subtypes of pathways that involve proteins such as Arf-6, RhoA, and Cdc42 [52]. Studies have shown that Arf-6-dependent CIE contributes to the endocytosis of the major histocompatibility complex (MHC) class I [53], the β-integrins [54], the glucose transporter GLUT1, and other proteins involved in amino acid uptake and cell–extracellular matrix interactions [55]. In addition, RhoA- and Cdc42-regulated endocytosis are dependent on lipid rafts for vesicle formation. RhoA is a dynamin-dependent pathway that has been described in the internalization of the β-chain of the interleukin-2 receptor (IL-2R-β) and other proteins in both immune cells and fibroblasts [56]. In contrast, Cdc42 is a dynamin-independent pathway described as a principal route for the uptake of cholera toxin B (CtxB) and the *Helicobacter pylori* vacuolating toxin (VacA) [57,58]. Cargos entering the cell through CIE are usually delivered to the early endosomes, followed by the transfer to late endosomes and lysosomes. In addition, cargo can be routed to the trans-Golgi network or recycled back to the plasma membrane [59]. CIE is the internalization route described preferentially for polyplexes of self-branched and trisaccharide-substituted chitosan oligomer nanoparticles (SBTCO) for the delivery of DNA [60] and for cowpea mosaic virus (CPMV), which has been extensively studied in recent years as a strategy for vaccine development, *in vivo* vascular imaging, and tissue-targeted delivery [61]. Recent studies suggest that CIE is involved in a novel mechanism for the uptake of nanoparticles that was described as a type of macropinocytosis. This new mechanism was found to be dependent on actin filaments and dynamin and has been compared to the action of an *excavator shovel* [62]. In another study, Garaiova et al. [63] showed that nanoparticles derived from trisaccharide-substituted chitosan oligomers (SBTCO) had higher uptake and better transfection efficacy than nanoparticles prepared from a linear chitosan (LCO). SBTCO were primarily taken up by cells via CIE and were successfully released from the endocytic vesicles. In contrast, LCO suspension in the cell culture medium resulted in nanoparticle aggregation and less cellular internalization compared to SBTCO nanoparticles.

2.2.3. Caveolae

Caveolae are flask-shaped invaginations (60–80 nm) of plasma membrane that participate in different cellular processes including cholesterol homeostasis, endocytosis of proteins, and signal transduction [64]. Caveolae are abundant in several types of cells, such as fibroblasts, smooth muscle, adipocytes, and endothelial cells, and are absent in neurons and leukocytes. Interestingly, in the case of adipocytes, caveolae can occupy as much as ~50% of plasma membrane [65], and the percentage of caveolae can be as high as ~70% of plasma membrane, as in the case of the endothelial cells in blood capillaries [66]. Initial studies have revealed caveolin (CAV1, CAV2 and CAV3) as the main protein constituent of caveolae, with an estimated of about 140–150 CAV1 protein molecules per caveola [67]. Cavins, the coat proteins (cavin 1–4), are known to work together with caveolins to regulate the formation of caveolae, and may also participate in the signals that regulate caveolae fate [68]. The intracellular destinations of caveolae have been the subject of controversy for many years. Nevertheless, it has emerged that in endothelial cells caveolae are able to perform transendothelial transport, which may be exploited for the release of nanoparticles in subendothelial tissues [69]. The material that is endocytosed via the caveolin-mediated pathway is initially localized into caveosomes, whose neutral pH avoids the hydrolytic environment of lysosomes. The sorting of caveosome cargo to the Golgi apparatus and endoplasmic reticulum may also be exploited for the targeted delivery of theranostic agents. Negative surface charges have been found to trigger cellular internalization predominantly via caveolae [91]. Liu et al. have employed the rabies virus glycoprotein RVG29 (29-amino-acid peptide) as a targeting moiety for a DNA-conjugated poly(amido amine) (PAMAM) dendrimer carrier that exhibited significant accumulation in the brains of mice after intravenous administration. The cellular internalization mechanism of PAMAM–RVG29 in the brain capillary endothelial cells was found to be through a combination of clathrin-

and caveolae-mediated energy-dependent endocytosis involving an interaction with the $GABA_B$ receptor [70]. Interestingly, previous studies of the mechanisms by which rabies virus glycoprotein crosses the blood-brain barrier (BBB) and achieves cellular internalization have revealed a specific interaction with the nicotinic acetylcholine receptor (AchR) [71]. This study suggests that RVG29-conjugated nanoparticles may be employed for crossing the BBB and delivering drugs to the brain.

2.2.4. Macropinocytosis

Macropinocytosis is an actin-driven endocytic process by which cells internalize considerable volumes of extracellular fluid through large vesicles (diameter of 0.5–10 μm) known as macropinosomes [72]. Macropinocytosis is a typical route for the uptake of apoptotic cell fragments [73], viruses [74] and bacteria [75] and contributes substantially to antigen presentation in major histocompatibility complex II (MHCII) [76,77]. Unlike receptor-mediated endocytosis and phagocytosis, the activation of macropinocytosis is not regulated by the direct action of a receptor or cargo molecules. In this case, the activation of a tyrosine kinase receptor (such as epidermal growth factor or platelet-derived growth factor receptor) leads to an increase in actin polymerization, actin-mediated ruffling, and macropinosome formation [78]. Interestingly, macropinosomes share some proteins (Cdc42, Arf6, and Rab5) with other endocytic processes, suggesting a relationship between the mechanisms of macropinosome biogenesis and other endocytic routes [79,80]. The macropinosomes are sensitive to cytoplasmic pH and undergo acidification and fusion events [81]. In macrophages, macropinosomes undergo a fate similar to that of endosomes, and during their maturation gain and lose markers that are typical for early and late endosomes before their fusion with lysosomes [82]. Micron-size particles are generally known to be internalized by cells via macropinocytosis [83]; however, most of the literature reports that nanoparticles undergo cellular internalization via more than one endocytic pathway. Recently, Zhang et al. [84] reported on lapatinib-loaded nanoparticles formulated with a core of albumin and a lipid corona formed by egg yolk lecithin. The nanoparticles exhibited a size of ~62 nm and a zeta potential of 22.80 mV and were internalized by BT-474 cells through energy-dependent endocytosis involving clathrin-dependent pinocytosis and macropinocytosis.

2.3. Differential uptake of nutrients in cancer cells—a novel gateway for nanomedicines

Essential nutrients such as lipids, free forms of fat-soluble vitamins, and carotenoids are transported across the plasma membrane by simple diffusion [85]. The transport of some nutrients such as glucose or lipoproteins is actively controlled by the endocytic pathways described earlier in this section [86,87]. The uptake of nutrients in normal cells is a well-regulated process, however; abnormally high cell proliferation rate (for example in the case of cancer progression) concomitantly triggers a higher rate of nutrient uptake. Several studies support the suggestion that the actively proliferating cells could adapt their endocytic mechanisms to meet the enhanced nutrient requirement. Recently, a study of human melanoma cells revealed significantly increased expression of the amino acid transporter for leucine (LAT1) and glutamine (ASCT2) [88]. Another study using a pancreatic tumor xenograft model suggests that macropinocytosis is an important route for nutrient uptake in tumor cells [89]. This study revealed that the enhanced rate of albumin uptake for meeting a higher glutamine demand of cancer cells (MIA PaCa-2 cells) is a functional contribution of the macropinocytosis stimulated by the oncogenic Ras proteins. These hyperactive endocytic mechanisms have not been explored in the context of nanomedicine and offer a potential strategy for enhancing the uptake of therapeutic nanoparticles by cancer cells. For any future development in this area, a deeper understanding of the differential physiopathological processes associated with both diseased and normal cells is a prerequisite to evaluate the relevance of differential nutrient uptake as a gateway for cellular internalization of nanomedicines.

Keeping in view the fact that the size, geometry, surface charge and mechanical properties of nanoparticles can influence cellular uptake efficiency, several studies have focused on optimizing physical and chemical attributes of nanoparticles to improve cellular uptake and consequently enhance pharmacological effects. These details are comprehensively covered in the several excellent review articles [11,90,91]. In addition to tuning the physical characteristics, receptor-mediated cellular internalization has also been extensively employed for enhancing cellular uptake of nanomaterials, and examples relevant to the field of nanomedicine are reviewed in the following section.

3. Receptor-mediated cellular internalization of ligand-targeted nanomedicines

The overexpression of receptors on the surface of target disease cells has been widely explored to improve the cellular uptake of nanoparticles as well as to minimize off-site toxicity. Representative examples of receptors known for active disease cell targeting include folate receptor (FR), transferrin receptor (TfR), epidermal growth factor receptor (EGFR), G-protein coupled receptor (GPCR), low-density lipoprotein receptor (LDLR), and lectins [92]. The receptor-mediated cellular internalization of nanoparticles has been studied for the intracellular delivery of various cargos such as small-molecule drugs, DNA, siRNA, and miRNA. Different types of high-affinity ligands, the moieties that specifically bind to a particular receptor, have been functionalized on the surface of nanoparticles to augment their interaction with cells, cellular uptake, and subsequent internalization. The major types of targeting ligands include high-affinity small molecules, peptides, aptamers, and antibodies. In the following section, recent developments related to targeting strategies using ligand-conjugated nanoparticles will be addressed highlighting FR, TfR, EGFR, PMSA, Integrin, and FcRn, receptors that have demonstrated potential for augmenting the field of nanomedicine.

3.1. Folate receptor (FR) targeting

The folate receptor (FR) is overexpressed in a variety of common tumor types (e.g., ovarian, lung, brain, and colorectal cancer) [93]. FR is a glycoprotein with molecular weight 38–44 kDa and exists in isoforms FR-α and FR-β. FR-α is expressed in various epithelial cancer types (e.g., ovarian 90%, endometrial 90%, brain 90%, and renal carcinomas 75%) as well as on some normal epithelial membranes such as those in the kidneys [94]. In contrast, FR-β is found on activated macrophages [95] and on the surface of hematopoietic malignancies such as chronic myelogenous leukemia [96]. For targeting of chronic inflammatory diseases such as type 2 diabetes, atherosclerosis, and rheumatoid arthritis, FR-β may prove to be an important target. Folic acid (441 Da) shows high affinity ($K_D = 10^{-9}$ M) to the FR, which allows the selective delivery of folate-conjugated nanocarriers to FR-expressing disease cells [97]. The use of small molecules like folic acid instead of peptides and antibodies as targeting ligands can provide several benefits. The main advantages include easy scale-up for clinical applications, facile chemical modification, no risk of toxicity or immune reactions due to its function as a vitamin, and high stability in acidic or basic media and at high temperature.

The concept and efficacy of folate targeting systems have been extensively studied *in vitro* and *in vivo* using folate-conjugated nanoparticles, chemotherapeutics, liposomes, and oligonucleotides [98]. PEGylated liposomal doxorubicin (DOX) containing folate-PEG-distearoyl-phosphatidyl-ethanolamine showed higher therapeutic activity than non-targeted DOX in the KB (human nasopharyngeal epidermoid carcinoma cell line) and KB-V (vincristine-resistant derivative) xenograft model and in the J6456 intra-cavitary therapy model, both of which overexpress FR [99].

Jiang et al. developed folate-conjugated human serum albumin nanoparticles loaded with docetaxel and investigated their antitumor activity in a human hepatoma cell line and in an *in vivo* model [100]. The folate-conjugated nanoparticles had superior antitumor activity based on *in vivo* inhibition ratios. Chemotherapeutic drugs loaded into folate-functionalized polymer-derived nanoparticles have been reported to exhibit significantly higher *in vivo* efficacy than non-targeted nanoparticle therapeutics as evaluated in an ovarian peritoneal metastasis model [101]. The high affinity of folate moieties is also of interest for use in enhancing tumor immunogenecity as well as targeting leukemia cells. Lu et al. were able to demonstrate that treatment with folate-conjugated hapten not only elicited more immunogenic tumor cells but also enhanced the anticancer immune reaction against hapten-treated tumor cells [102]. In targeting acute myelogenous leukemia (AML) blast cells with overexpressed FR-β (70%), folate-conjugated liposomal DOX more strongly inhibited colony formation than the non-targeted analog in MV4-11 (human acute myelocytic leukemia) and K562 (human erythromyeloblastoid leukemia) cells as well as AML patient cells [103]. These studies suggest the efficacy and the potency of folate targeting in pre-clinical as well as clinical applications.

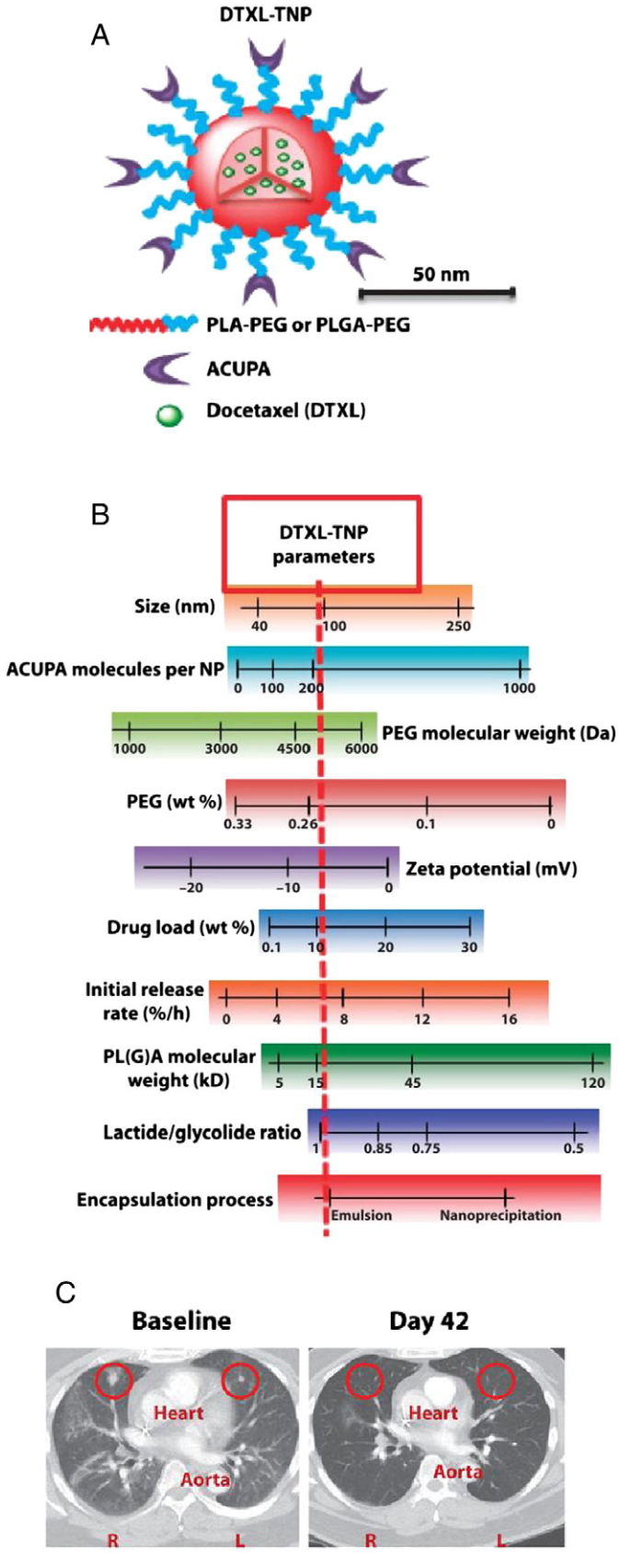
3.2. Transferrin receptor (TfR) targeting

The transferrin receptor (TfR), which is a cell membrane glycoprotein with a homodimer of two identical transmembrane subunits, mediates cellular uptake of iron from a plasma glycoprotein (i.e., transferrin) [104]. The two subunits of TfR are each of 84,910 Da and have 760 amino acid residues, notably three N-linked glycosylation sites and an O-linked glycosylation site, which are necessary for normal function of the receptor [105,106]. The main function of TfR is to regulate cell growth and the cellular uptake of iron from transferrin (~80 kDa) [107]. Though TfR is probably expressed on all cells, its level of expression varies. TfR is highly expressed in immature erythroid cells, placental tissue, rapidly dividing cells, and is about 100-fold higher in cancer cells than normal cells [108]. This makes TfR one of the most attractive targets for cancer therapy by receptor-mediated endocytosis of drug nanoparticles. Transferrin (Tf) and TfR binding single-chain antibody fragment (TfRscFv) have been used as ligands for transferrin receptor-mediated intracellular delivery of nanotherapeutics. Examples of TfR-targeted nanomedicines currently in various stages of clinical trials include CALAA-01 (a four-component nanomedicine composed of Tf-functionalized PEG, Tf- and adamantane-functionalized PEG, SiRNA, and a cyclodextrin-bearing polymer, in Phase I clinical trials), MBP-426 (liposome-based, oxaliplatin-loaded, Tf-conjugated nanomedicine in Phase II clinical trials), SGT-53 (intracellular delivery of p53 plasmid DNA using TfRscFv-conjugated liposome-based nanomedicine in Phase Ib clinical trials), and SGT-94 (RB94 plasmid DNA delivery using TfRscFv-conjugated liposome, in Phase I clinical trials) [109]. These successful examples have garnered substantial interest in the further development of TfR-targeted nanomedicine. Choi et al. reported on PEGylated gold nanoparticles with different amounts of human transferrin for active targeting leading to efficient nanoparticle internalization into TfR-overexpressing cancer cells (Neuro2A cells) as well as in the tumor site in Neuro2A tumor-bearing mice, suggesting better intracellular delivery of therapeutic agents to the target cancer cells [110]. For effective treatment of brain glioma cells, transferrin-conjugated magnetic silica PLGA nanoparticles containing anticancer drugs (DOX and/or paclitaxel) were evaluated in intracranial U-87 MG-luc 2 xenograft mice. The targeted nanoparticles had greater anti-glioma activity than non-targeted nanoparticles [111]. The TfR-mediated internalization of anticancer drugs into brain tumor tissue has been associated with the highly overexpressed TfR in brain capillary endothelium and glioma cells. In addition, Hong et al. showed that the endocytosis of Tf-PEG-niosomes by KB cells was inhibited by low temperature and by free Tf, indicating the high specificity towards TfR [112]. As expected, the hydroxycamptothecin (HCPT)-loaded Tf-PEG-niosomes produced the strongest cytotoxicity to three carcinomatous cell lines (KB, K562, and S180) and significantly stronger inhibition (71%) of tumor growth in S180 tumor-bearing mice compared to non-targeted PEG-niosomes. Overall, these studies represent the efficacy of Tf targeting using various nanoparticles (e.g., metal, polymer, and liposome-based carriers) *in vitro* and *in vivo*. A recent study by Salvati et al. [113] reports the loss of targeting ability of Tf-functionalized nanoparticles in biological media. The protein constituents of the biological media were believed to neutralize TfR targeting of Tf ligands covalently localized at the surface of nanoparticles through a PEG linker. This finding is of paramount importance and calls for the evaluation of any potential targeting nanomedicine in biological medium rather than buffered saline. It further highlights the importance of optimizing ligand density to balance desired systemic circulation with targeted cellular uptake.

3.3. Epidermal growth factor receptor (EGFR) targeting

The epidermal growth factor receptor (EGFR, also known as HER1) is a member of the ErbB tyrosine kinase family, which also includes HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [114,115]. EGFR, which is overexpressed in various solid tumors including colorectal, brain, breast, ovarian, pancreatic, and prostate cancers, can stimulate tumor growth, invasion, and metastasis [116]. Both small molecules and monoclonal antibodies have been used as EGFR-targeting ligands; e.g., epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor, epigen, betacelluin, and epiregulin [117]. Upon binding of ligands to EGFRs, the homodimerization or heterodimerization of the monomeric EGFR occurs with other members of the ErbB family receptors and other cell-surface tyrosine kinases for cellular signaling.

Anti-EGFR ILs-DOX (Fab fragments of anti-EGFR antibody cetuximab-conjugated, DOX-loaded immunoliposomes targeted to EGFR in Phase I clinical trial) is a notable example of EGFR-targeted nanomedicines [109]. In a recent attempt at targeting HER1, Shevtsov et al. developed superparamagnetic iron oxide nanoparticles conjugated with recombinant human epidermal growth factor (SPION-EGF) to improve magnetic resonance imaging of malignant brain tumors and demonstrated more efficient tumor imaging than non-targeted SPION in an orthotopic model of C6 gliomas [118]. In addition, the use of cisplatin-encapsulated gelatin nanoparticles with EGF improved *in vitro* and *in vivo* targeting ability and anticancer effects in A549 cells and a tumor-bearing mice model (high EGFR expression) compared to HFL1 cells (low EGFR expression) [119]. For targeting HER2 (which accounts for 14–91% of breast cancer cases), the monoclonal antibody trastuzumab (also known as Herceptin) is now widely used for patients with HER2-positive breast cancers [120]. In pre-clinical studies, Lee et al. demonstrated that DOX-loaded PEG-PLGA-Au half-shell nanoparticles with Herceptin accumulated more quickly in tumor sites in mice bearing SK-BR-3 (breast cancer cell line with high HER2 expression) by receptor-mediated endocytosis and eventually achieved stronger therapeutic efficacy based on chemotherapy and hyperthermia compared to non-targeted nanoparticles [121]. MM302 (HER2-targeted, scFv antibody fragment-conjugated, DOX-loaded liposome) is an example of a HER2-targeted nanomedicine in Phase I clinical trial [109]. HER3 (the only member of the EGFR family lacking intrinsic tyrosine kinase activity), has recently gained significant interest for targeting EGFR [122]. The fully humanized HER3 antibody U3-1287 (AMG 888) reduced growth of cancer cells and improved tumor suppression in a xenografted human HNSCC FaDu model. Therefore, based on these research results, cellular targeting strategies involving receptor-



mediated endocytosis are likely to reach clinical application in the treatment of various diseases, especially cancers.

3.4. Prostate-specific membrane antigen (PSMA) targeting

The type 2 integral membrane glycoprotein PSMA is a valid cancer target overexpressed on the surface of prostate carcinomas and the neovasculature of a majority of solid tumors [123,124]. In this regard, a notable example is BIND-014, docetaxel-loaded, polymer (PLA-PEG and PLGA-PEG)-based nanomedicine designed for targeted delivery by employing a small-molecule ligand (S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid, ACUPA) against PSMA [18]; BIND-014 is currently in Phase II clinical trials. A combinatorial approach, screening about 100 formulations, was employed to select desirable nanomedicine attributes including particle size, targeting ligand density, surface hydrophilicity, drug loading, systemic circulation, and drug release properties (Fig. 2). This thorough optimization yielded a highly effective PSMA-positive prostate cancer-targeted nanomedicine with a remarkable tumor shrinkage in humans at doses below the typical clinical doses of solvent-based docetaxel formulations (Fig. 2).

3.5. Integrin targeting

Integrins are a family of heterodimer transmembrane receptors involved in a number of vital cellular functions (adhesion, migration, invasion, stress responses, proliferation, differentiation, survival, and apoptosis) through modulating endothelial cell–extracellular matrix interactions and regulating intracellular signaling [92]. Among more than 24 surface receptors derived from the dimerization of about 18 α and 8 β subunits, $\alpha_v\beta_3$ receptor was found to be differentially overexpressed in tumor-related endothelial cells during angiogenesis compared to - was found to be endothelial cells in normal tissues [125–128]. Various strategies have been employed to develop high-affinity ligands for $\alpha_v\beta_3$ receptor-targeted nanomedicines, and cyclic-RGD peptides have emerged as the most promising targeting ligand [92,129–131]. A recent report from Graf et al. [132] employed Pt(IV) prodrug-loaded PLGA-PEG nanoparticles functionalized with cyclic-RGD for $\alpha_v\beta_3$ receptor-targeted delivery. The encapsulated drug was found to be more efficacious with higher tolerance compared to cisplatin in an *in vivo* orthotopic human breast cancer xenograft model. There are no examples of liposome- or polymer-based, integrin-targeted nanomedicines in clinical trials.

3.6. Neonatal Fc-receptor (FcRn) targeting—an avenue to oral delivery of nanomedicine

Nanomedicine administration is currently limited to parenteral routes. However, for convenience and optimal patient compliance, the oral route of administration is of particular interest, especially for treatment of diseases that require frequent administration [133]. Nevertheless, oral administration is limited by poor intestinal absorption of nanomaterials because of their inability to cross the intestinal epithelium cellular barrier [134]. It has been reported that FcRn in neonatal intestine is responsible for safe transport of breast milk immunoglobulin (IgG) to offspring, offering a potential means to overcome the intestinal epithelium cellular barrier [135]. In adults FcRn receptor expression in the apical region of epithelial cells of the small intestine is equivalent to neonatal expression. Interestingly, the affinity between the Fc region

Fig. 2. (A) Schematic illustration of PSMA-targeted BIND-014 docetaxel-loaded polymeric nanoparticles. (B) Depiction of achievable nanomedicine attributes; red line represents the optimized properties. (C) CT scan evidencing a remarkable regression of lung metastases in 51-year-old male patient treated with two cycles of BIND-014.
Reproduced with permission from [18].

of IgG and FcRn is pH dependent; i.e., there is higher binding affinity at acidic pH (<6.5) compared to physiological pH (~7.4). Capitalizing on these facts, Pridgen et al. [136] conducted a groundbreaking proof-of-concept study demonstrating the unprecedented potential of FcRn targeting as an avenue to oral delivery of nanomedicine. PLA-PEG polymer nanoparticles surface-functionalized with polyclonal IgG Fc fragment (NP-Fc) exhibited transepithelial transport both *in vitro* and *in vivo*. Oral administration of fluorescently labeled NP-Fc in fasted wild-type mice revealed the ability of Fc-functionalized nanoparticles to cross the intestinal epithelial barrier and enter the lamina propria (Fig. 3). The efficiency of absorption for targeted nanoparticles was 11.5 times higher than that of nontargeted particles. The particles also localized in other organs, which shows that they were able to enter systemic circulation (Fig. 3). Furthermore, oral administration of insulin-loaded NP-Fc to fasted wild-type mice resulted in a hypoglycemic response. A control experiment with FcRn knockout mice elicited no such hypoglycemic response, which established that the superior outcome is because of FcRn targeting. Although this novel avenue is still in its infancy, the initial results are promising enough to expect an impact on future development of nanomedicines that can be administered orally.

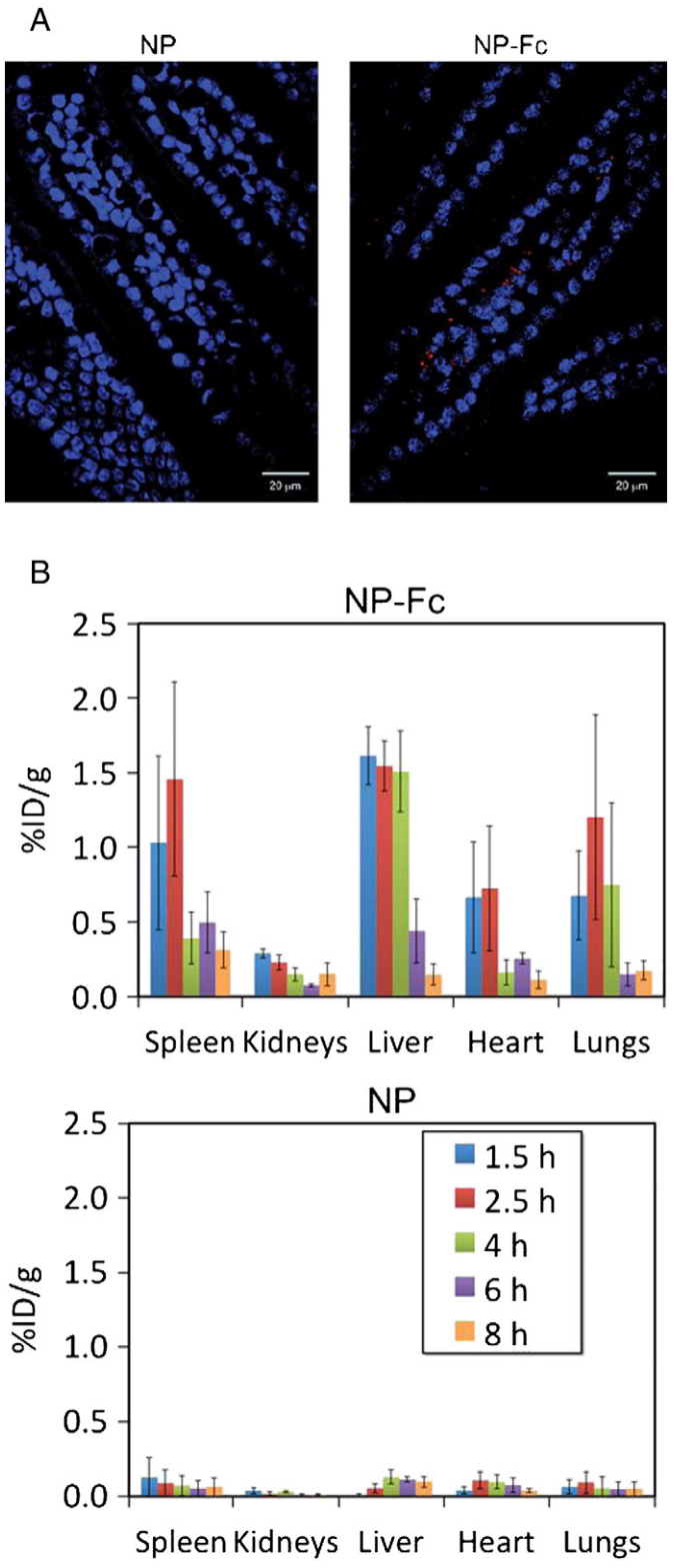


Fig. 3. (A) FcRn-targeted nanoparticles can be seen as red puncta in the confocal fluorescence images of sections of mouse duodenum. (B) Comparison of organ localization of FcRn-targeted (top) and non-targeted (bottom) PLA-PEG nanoparticles. Reproduced with permission from [136].

4. Intracellular trafficking and subcellular targeting

4.1. From endosomes/lysosomes to cytoplasm

Ligand-conjugated nanoparticles with specific tissue- or cellular-level targeting have already been successfully produced, and this novel paradigm combines with the EPR effect to increase intratumoral concentration of cytotoxic anticancer drugs. Many interesting therapeutic targets are localized in the intracellular compartments. This fact has triggered increasing efforts to develop sophisticated nanoparticle designs capable of precise navigation across physiological barriers and selective delivery of therapeutic and diagnostic agents to intracellular targets [137]. Despite different cellular entry pathways, endosomes are the first intracellular compartments encountered by the internalized nanoparticles. Early endosomes are characterized by a lower luminal pH (~6–6.5, necessary for the activity of endosome-specific enzymes), which has been exploited to trigger the release of pH-responsive nanoparticles into the cytosol. Polyamine-based carrier systems can efficiently achieve neutralization after being protonated under the acidic conditions of the endosome. This neutralization increases endosomal pH, which triggers ionic transport into the lumen of the endosome, resulting in swelling and possible release of endocytosed nanoparticles (proton sponge effect) [138]. The choice of material to induce the proton sponge effect has been limited to polyamines. However, these materials suffer from poor biocompatibility and high toxicity inherent in the high pKa (~9) of amine groups, which can induce membrane lysis at acidic as well as physiologic pH. Measures reported to reduce the toxicity of the polymer system include the protection of amine groups with acid-sensitive groups that cleave and expose the amino groups only under endosomal pH and incorporation of low-pKa heterocycles as pendant groups [139]. A timely response to the pH is crucial, as failing to respond efficiently can delay release, resulting in either transfer of endocytosed nanoparticles to hydrolytic-enzyme-containing lysosomes for degradation or their direction to the plasma membrane via a recycling route.

Intracellular spatiotemporally controlled trafficking and the fate of endocytosed cargo are regulated by RAB proteins (one of the five major subfamilies of the superfamily of G-proteins) in combination with a number of effectors or regulatory molecules. In cancer progression, increased activity of the RAB proteins has been associated with an increased rate of growth factor receptors being recycled to the plasma membrane. Therapeutic interventions to modulate RAB activity offers interesting and rather less explored potential targets [140,141].

Well-regulated endocytic and endosomal trafficking are of paramount importance, as dysfunctional trafficking has been linked to neurodegenerative diseases (e.g., Alzheimer's disease, Huntington's disease,

autism) [142–145]. Na-H exchangers (NHEs) are a family of nine Na^+/H^+ exchanger isoforms found in mammals. NHE6 has been found in endosomes from a number of different cell types and is associated with the regulation of lumen pH in early, late, and recycling endosomes as well as lysosomes, where pH decreases to 5 [146,147]. In mature endosomes, spatiotemporal control of the lumen pH is essential to control the degradation rate and the recycling time of the internalized material (dissociation of ligand–receptor conjugates and recycling of the receptors to the plasma membrane). Some NHEs (NHE1–NHE5) are localized in plasma membrane and play a crucial role in the initiation and development of cancer [148]. Interestingly, the hydrogen gradient of all malignant tumors is reversed compared to normal tissues. The intracellular pH of tumor cells is alkaline (7.12–7.7), whereas the pH in the extracellular environment is acidic (6.2–6.9) [149,150]. Maintenance of such a distinct hydrogen ion dynamic has been attributed to the abnormally high activity of NHE1, which is essential for the survival of cancer cells in the hostile extracellular environment. Dysfunctional pH control has also been associated with the development of multidrug resistance. While maintaining an alkaline cytoplasmic pH, NHEs play an important role in maintaining a highly acidic pH in the lumens of intracellular organelles. NHE1 inhibitors are now being employed as selective tumor drugs [34,151,152]. The field of nanomedicine is yet to contribute in this context, and these attributes should be considered while developing nanoparticles for cell membrane, endosome, and lysosome targeting.

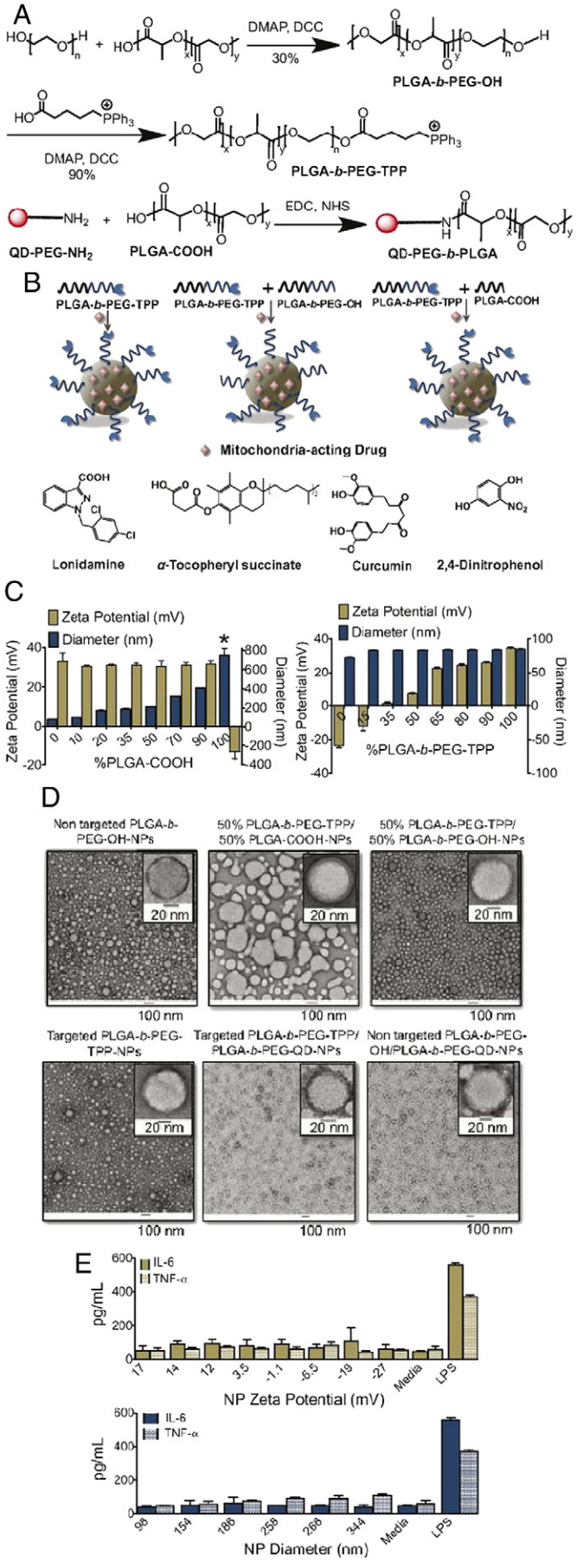
Lysosomes are considered digestive or recycling components of the cell machinery because they contain cathepsin proteases. The degradation products resulting from the enzymatic activity on the cargo materials are released into the cytoplasm to meet the nutritional needs of the cell. The tumor cells exhibit higher activity of lysosomal cathepsin, and its release into the extracellular environment has been implicated in the promotion of tumor growth. The concept of lysosomotropism was introduced by Christian de Duve, who referred to lysosomes with their hydrolytic enzymes as “suicide bags” [153]. Since then the destabilization of lysosomes via lysosomal membrane permeabilization (LMP) and release of their hydrolytic contents in the cytoplasm as a means of triggering cell death have been highlighted as potential targets for therapy. However, multidrug resistance acquired by the cancer even in the early stages of its development renders common LMP-triggering stimuli irrelevant. Consequently, more potent lipophilic amine-based cationic amphiphiles are being employed as lysosomotropic detergents. Recently, inhibiting the role of acid sphingomyelinase (ASM) in supporting lysosomal membrane integrity was exploited to induce LMP using cationic amphiphilic drugs (CADs). For the several cancer cell types tested, CADs killed cells at concentrations much lower than the concentrations that induce cytotoxicity in normal cells [154–156].

In the event that the endosome or lysosome is not the final therapeutic target, it is necessary to devise a strategy for endosomal release and for protecting the administered drug from degradation in the hostile lysosomal environment. This is being achieved by encapsulating drugs into a variety of nanoparticle-based carrier systems. These nanoparticles not only protect the sensitive drug molecules from degradation but also release the drug in a time-dependent manner [157]. Excessively delayed release may expose the drug to the aggressive endosomal or lysosomal environment (low pH and hydrolytic enzymes), leading to the loss of any therapeutic impact. Polymer nanoparticles and liposomes are being widely explored as biocompatible, bioresponsive, and biodegradable nanocarrier systems. Various strategies have been explored to effect the release of a cargo from the endosome into the cytoplasm. These include the use of cell-penetrating peptides, stimuli-responsive polymers (endoosmolytic mechanism to induce endosomal disruption and release by exploiting differential redox or pH environment), or fusogenic liposomes (fusogenic mechanism: emptying the particle in cytoplasm during the fusion of liposomal particles with the endosomal membrane) [158]. Zheng et al. have reported pH-responsive nanoparticles derived from PEGylated polyphosphazene laterally functionalized with *N*,*N*-diisopropylethylenediamine (DPA) [159]. The DPA units imparted pH-triggered release capability to the resulting nanoparticles, as demonstrated by loading and release of DND-26, a fluorescent dye that preferentially accumulates in acidic compartments in a cell. DND-26 was found to be distributed in the whole cell rather than being concentrated in the endosome and lysosome when cells were exposed to the free DND-26. DPA's functional group is of particular interest because of its distinct pKa value (6.3) as compared to the pKa (7.4) of *N*,*N*-dimethylethyleneamine. Careful selection of such functionalities can modulate payload release from nanoparticles at a particular pH [160]. Another pH-responsive nanoparticle platform, derived from a block copolymer of PEG and polyamide, where the polyamide block is laterally functionalized with citraconic amide and succinic amide, was reported by Lee et al. [161]. The interesting feature of this platform is the degradation of the citraconic amide at the endosomal pH, leading first to side chain degradation, then polymer charge reversal and consequently destabilization of nanoparticles and release of loaded lysozyme. Despite several reports on stimuli-responsive materials, there are hardly any nanoparticle platforms at the clinical stage, which highlights the need to further bridge the gap between different disciplines actively working on the development of materials for biomedical applications. Binauld and Stenzel [162] have comprehensively reviewed the polymeric materials that can degrade under acidic pH, which should be of help in designing clinically relevant polymers.

The cytoplasm is home to a wide range of biological processes identified as therapeutic targets for many diseases. Transporting nanotherapeutics to the cytoplasm has mainly been through the endosomes; however, some cell-penetrating peptides (CPP) can enter the cytoplasm by directly traversing the plasma membrane. The ability of CPP to directly translocate across the plasma membrane is greatly reduced when conjugated with a cargo [163,137]. Some CPPs have, in fact, been employed as anticancer agents per se (e.g., Azurin against solid tumors under Phase I clinical trials and XG-102 targeting c-Jun-N-terminal kinases under Phase II clinical trials). Co-administration of a CPP (iRGD) via systemic injection has resulted in improved therapeutic index of a variety of drug entities (DOX, nab-paclitaxel, DOX liposomes, and trastuzumab) [164]. The underlying mechanism of this effect stems from the ability of the iRGD to bind to αv integrins, which are exclusively expressed on the tumor vessel endothelium. After preferentially homing to the tumors, iRGD is proteolytically transformed into CRDGK/R, which loses its affinity with the αv integrins but acquires affinity to the neuropilin 1 (NRP-1), triggering tissue penetration by enhancing vascular permeability. The whole process is tumor specific because of the αv integrin binding specificity of iRGD. The iRGD-assisted improved therapeutic index has been demonstrated only in mouse tumor models, and the efficacy of this platform has yet to be shown in human patients.

4.2. Endoplasmic reticulum and Golgi apparatus

An alternate route to cellular entry that avoids the acid pH and hydrolytic lysosomal environment is the retrograde trafficking pathway, which leads the endosomal cargo to the Golgi apparatus (GA) and endoplasmic reticulum (ER) [165]. The ER and GA are responsible for calcium homeostasis, folding of membrane and secretory proteins, and lipid biosynthesis. The retrograde trafficking pathway is involved in the recycling of certain receptors (e.g., mannose-6 phosphate receptor) and is exploited by certain toxins (e.g., ricin toxin, shiga toxin, anthrax toxin lethal factor, and cholera toxin) for localizing in and interfering with the function of the ER [166,167]. An irregular ER function referred to as ER stress results in the unfolded protein response (UPR), which leads to protein synthesis inhibition, refolding of proteins, and clearance



of misfolded proteins. ER stress can lead to apoptosis, a cause of heart diseases (cardiac hypertrophy, degeneration of cardiomyocytes), liver diseases, neurodegenerative diseases, and diabetes [168,169]. UPR has also been proposed as an anticancer target because of its critical role in tumor cell resistance to hypoxia and tumor progression [170]. A nanoparticle platform capable of targeting ER stress or UPR is still awaited, though there are some notable examples in the literature that may lay a foundation for such an endeavor. In an *in vitro* experiment, nanocarriers based on PLGA (95 nm ± 20) were found to accumulate predominantly in the Golgi apparatus in the case of human bronchial epithelial (HBE) and opossum kidney (OK) renal tubule cells, as revealed by immunofluorescent compartment labeling [171]. Remarkably more particles were localized in the GA as compared to the lysosomes. The authors proposed that the PLGA particles were able to avoid localization in late endosome or lysosomes and were apparently transferred from the early endosome to the GA via ER. The presence of albumin in the culture medium was suggested to have coated the nanoparticles, resulting in receptor-mediated cellular internalization and subsequent association with the GA via a retrograde route. This study was not able to determine whether the particles are within the GA or are localized in the Golgi-associated vesicles of late endosomes. In a later study employing Raman spectroscopy in combination with optical microscopy, it was revealed that the PEO-functionalized PCL and PLGA nanoparticles were incorporated into the Golgi-associated vesicles of late endosomes in human HeLa cells (cell line CCL-2) [172]. The cellular internalization pathways can determine the intercellular destination of nanoparticles, and nanoparticles can be internalized via more than one pathway. The physiochemical nature and cell type used can influence the preference for a particular internalization pathway. PLGA nanoparticles internalized by MDCK epithelial cells were predominantly transported to the lysosomes, with some particles found in the ER and Golgi complex, emphasizing the critical importance of the internalization pathways [173]. The extent of nanoparticle uptake, mechanism of cellular uptake, and subsequent localization in subcellular compartments can vary with cell type for the same type of nanomaterial.

4.3. Mitochondria

Mitochondria are unique among cellular organelles, as they have a two-membrane structure (inner and outer mitochondrial membranes) with the mitochondrial DNA enclosed in the inner membrane. The protein constituents of the mitochondria are genome-coded proteins or proteins of nuclear origin. Mitochondrial diseases, therefore, originate from defective nuclear and mitochondrial genome. Mitochondrial dysfunction has been implicated in a number of diseases, including cancer, neurodegenerative and neuromuscular diseases, obesity, and diabetes, and has been recognized for offering important therapeutic targets [174–178]. A synergy of highly dense inner membrane (abundant in saturated phospholipids) and high membrane potential (negative inside) compared to the plasma membrane results in highly controlled transportation across the mitochondrial membranes [179,180]. Because of the highly selective and impermeable nature of the mitochondrial membrane, targeting and delivering therapeutic agents to mitochondria has been a formidable challenge. Cations are generally known to target the mitochondria primarily because of the high membrane potential [181–183]. Among different cations, triphenylphosphonium (TPP) cation [184], meets the prerequisites for transport: a balance between delocalized positive charge and lipophilicity. TPP cation has been shown to

Fig. 4. (A) Synthesis of TPP end-capped and QD-functionalized PLGA-PEG block copolymers. (B) Schematic illustration of drug-loaded targeted nanoparticles. (C) Modulation of size and zeta potential. (D) TEM images of the fabricated nanoparticles. (E) IL-6 and TNF-α secretion profiles of nanoparticles with different zeta potentials (top) and diameters (bottom).
Reproduced with permission from [196].

successfully cross this barrier and reach the inner leaflet of the inner mitochondrial membrane. Consequently, TPP has been exploited as a vector for delivering covalently conjugated small-molecule-based drugs to mitochondria [185–188]. The targeted delivery of therapeutic agents to mitochondria using a liposome-based nanocarrier platform has been demonstrated. After a series of attempts, TPP has been incorporated in the lipid bilayer membrane of liposomes by covalent conjugation with stearyl moieties. The resulting nanocarrier exhibited efficient delivery of anticancer drugs (sclaeol and ceramide) to mitochondria [189–191]. In addition to TPP, the octaarginine-functionalized liposome has also been found to deliver cargo to the mitochondria [192]. Depending on the surface density of octaarginine, the liposomes exhibited different cellular internalization pathways. For high octaarginine surface density, the liposomes efficiently escaped through macropinocytosis into the cytosol [193], whereas given a low octaarginine surface density, liposomes were internalized via clathrin-mediated endocytosis followed by transfer to lysosome for degradation [194]. Despite recent efforts showing some improvement, concerns persist regarding the immune response and *in vivo* toxicity associated with these targeting platforms [195,192]. Compared to liposomes, polymer-based nanocarriers for mitochondrial targeted delivery are rather less frequent. In a recent attempt Marrache et al. [196] reported a TPP end-functionalized PLGA-PEG (PLGA-PEG-TPP) nanoparticle-based delivery system for targeted delivery of various mitochondrial-acting drugs (Fig. 4). In order to optimize the particle size and surface charge for maximal mitochondrial uptake, they prepared various formulations by blending different ratios of PLGA-b-PEG-TPP with −COOH end-functionalized PLGA (PLGA-COOH) or with hydroxy end-capped PLGA-PEG (PLGA-b-PEG-OH). By varying the ratios between these polymers, the authors prepared nanoparticles of different sizes (ranging from ~80 nm to ~400 nm) with a constant surface charge (~+30 mV) and nanoparticles of approximately the same size but with different surface charge (ranging from ~−25 mV to +30 mV). Compared to the control nanoparticles with negative surface charge, the positively charged nanoparticles exhibited greater accumulation in the mitochondria of human cervical cancer (HeLa) cells. Interestingly, the positively charged targeted nanoparticles were able to escape from early endosomes and become localized in the mitochondria, whereas the negatively charged non-targeted nanoparticles were still found only in the endosomes even after 4 h incubation. Authors attributed this observation to the buffering effect of positively charged nanoparticles that can prevent acidification of endosomal vesicles, which may increase the ATPase-mediated influx of protons and counter ions. The resulting osmotic swelling ruptures the endosomal membrane, leading to cargo release in the cytosol. In an attempt to demonstrate the non-aggregating behavior of positively charged nanoparticles generally induced by negatively charged serum proteins, the authors further assessed the stability of the nanoparticles by monitoring changes in their size in the cell culture media: 10% (vol/vol) FBS in DMEM or 10% (vol/vol) FBS in H_2O for 7 days. The charged nanoparticles below 200 nm did not induce an immune response when tested for the production of proinflammatory cytokines (IL-6 and TNF-α) in RAW 264.7 macrophages by ELISA. The formulations were prepared by encapsulation of mitochondrial-acting drugs, including lonidamine and α-tocopheryl succinate for cancer (HeLa cells), the mitochondrial antioxidant curcumin for Alzheimer's disease (human neuroblastoma IMR-32 cells), and the mitochondrial uncoupler 2,4-dinitrophenol for obesity (3T3-L1 cells). The PLGA-b-PEG-TPP nanoparticles markedly improved the therapeutic index of all the employed drugs when compared to the non-targeted nanoparticles or the drugs alone. In a similar attempt Wang et al. demonstrated a preferential mitochondrial localization of TPP-functionalized nanoparticles derived from poly-L-lysine (PLL) [197]. Although TPP-functionalized nanocarriers have shown promising *in vitro* results, the *in vivo* performance of these materials needs to be evaluated for potential clinical applications.

4.4. Nucleus

The nucleus, an organelle wrapped in a double lipid bilayer containing important therapeutic targets (proteins, nuclear receptors, and DNA) for a variety of diseases, has been the focus of targeted delivery of both drugs and DNA (as a drug for gene therapy). In most instances, the nanoparticles deliver the drugs into the cell and the drug molecules diffuse through the cytosol to reach the nuclear target. Some reports have suggested an improved accumulation of the therapeutic agents in the nucleus as a result of tuning the molecular design of polymeric material employed for the fabrication of nanoparticles-. Polyplexes derived from copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and methacrylamide monomers bearing pendant L-lysine-based peptide groups with different numbers of L-lysine repeat units were reported to exhibit an improved nuclear accumulation (plasmid DNA delivery) when the number of L-lysine repeat units was ten (pHK10 and pHK15 stand for polymers with 10 and 15 L-lysine repeat

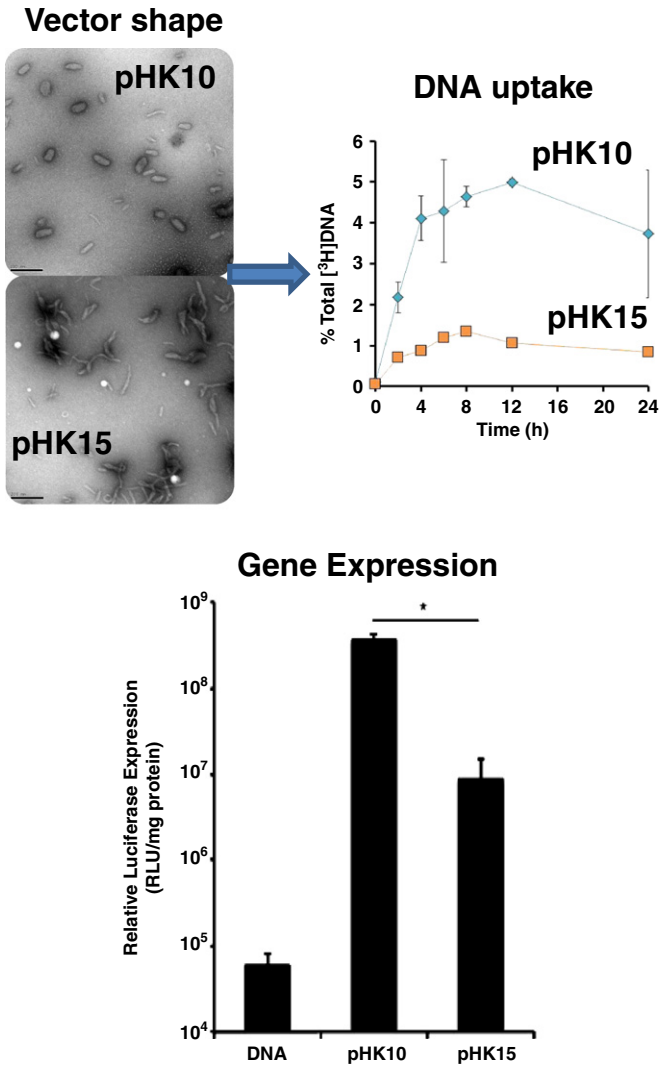


Fig. 5. TEM images revealing the effect of the number of L-lysine units on the vector shape (top-left). A comparison of DNA uptake capacity of pHK10 and pHK15 (top-right) and gene expression (bottom).
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units, Fig. 5) [198]. pHK10 copolymer interacted with the plasmid DNA, resulting in nanoparticles with a lower aspect ratio (width ~25 nm and length ~74 nm) compared to nanoparticles (width ~18 nm and length ~102 nm) formed by the copolymer pHK15. A higher aspect ratio was associated with poor internalization and higher localization into the endosomes and lysosomes, thus delaying the nuclear delivery. Interestingly, the particles were reported to be internalized via caveolin-mediated endocytosis.

Caveolin-mediated endocytosis is of particular interest, as this cell internalization pathway delivers the endocytosed material to caveosomes/caveolae that have neutral pH, can bypass the acidic and enzymatic degradation in lysosomes, is known for sorting the cargo to the GA and ER that reside in close proximity to the nucleus, and has been proposed to provide nanomedicines with a safe route to the nucleus [199–201]. Certain functionalities such as L-arginine [202] and saccharide [203] moieties as constituents of carrier polymer systems have been reported to sort the polyplexes to the GA and ER. The saccharide functionalization strategy mimics the natural process of transporting the glycosylated proteins from the ER to the nucleus [204,205]. Further studies focusing on exploiting this avenue by developing new polymers with glycosylated or arginine functional groups may provide a convenient way of caveolae-mediated nucleus targeting. In a similar vein, β-cholanic acid-based hydrophobically modified glycol chitosan polymer nanoparticles (~359 nm) were found within the cytoplasm of HeLa cells after just two minutes of incubation with almost no localization in the lysosomes. About 20% of the particles were found localized in the lysosomes after 60 min of incubation, with most of the particles accumulating in the perinuclear region [206]. This approach has an inherent limitation of low efficacy, considering the amount of drug able to reach the nucleus. If the drug resistance machinery of the cell has been activated, the drug molecules have even less chance to reach the target. However, establishing a strategy to navigate through the cytosol and reach the nuclear target precisely is a nontrivial task. A more precise understanding of the transport mechanisms active in the cytosol would be helpful for designing intelligent carrier systems for delivery of cargo to specific targets.

After the plasma membrane barrier, the nuclear envelope presents another barrier to nuclear delivery. Transportation across the nuclear envelope occurs through the nuclear pore complexes (NPCs, Fig. 6), the perforations in the nuclear envelope. The NPC constituent proteins, nucleoporins (Nups), determine the NPCs' assembly, structural, and functional aspects. Nature employs two modes of transportation for trafficking across the NPCs, passive and active. Small ions and macromolecules (~9 nm) are transported across the nuclear envelope via passive diffusion through the NPCs, while macromolecules larger than 40 kDa (39 nm) are sorted via nuclear transport receptor-mediated active transport that is facilitated by the oligopeptide sequences specifically binding to the receptors, known as nuclear localization signals (NLSs). The FG-Nups constituent of NPCs contains phenylalanine-glycine (FG) domains, which are reported to line the inner NPC channel while extending on both sides of the nuclear envelope. Multiple, stochastic, low-affinity interactions between the transport receptors and FG-Nups play an important role in creating a barrier to translocation of cargo across the NPC [207].

The conjugation of nuclear localization signal (NLS) to nanoparticle-based carrier systems has been demonstrated to direct the cargo to the nuclear target. Cheng et al. [208] demonstrated that nanoparticles derived from NLS (CGGGPKKKRKVGG)-functionalized PLGA nanoparticles (~72 nm) and NLS-functionalized quantum dot-conjugated PLGA nanoparticles (~168 nm) were localized in the nucleus of HeLa cells. In a similar vein, NLS-functionalized DOX-loaded PLGA nanoparticles (~226 nm) were shown to deliver much more of the drug to the nucleus (MCF7 cells) when compared to the free drug of PLGA nanoparticles lacking an NLS. Conjugation with NLS further enhanced the cell cycle (G2/M phase) blocking capacity and induced a greater extent of apoptosis [209].

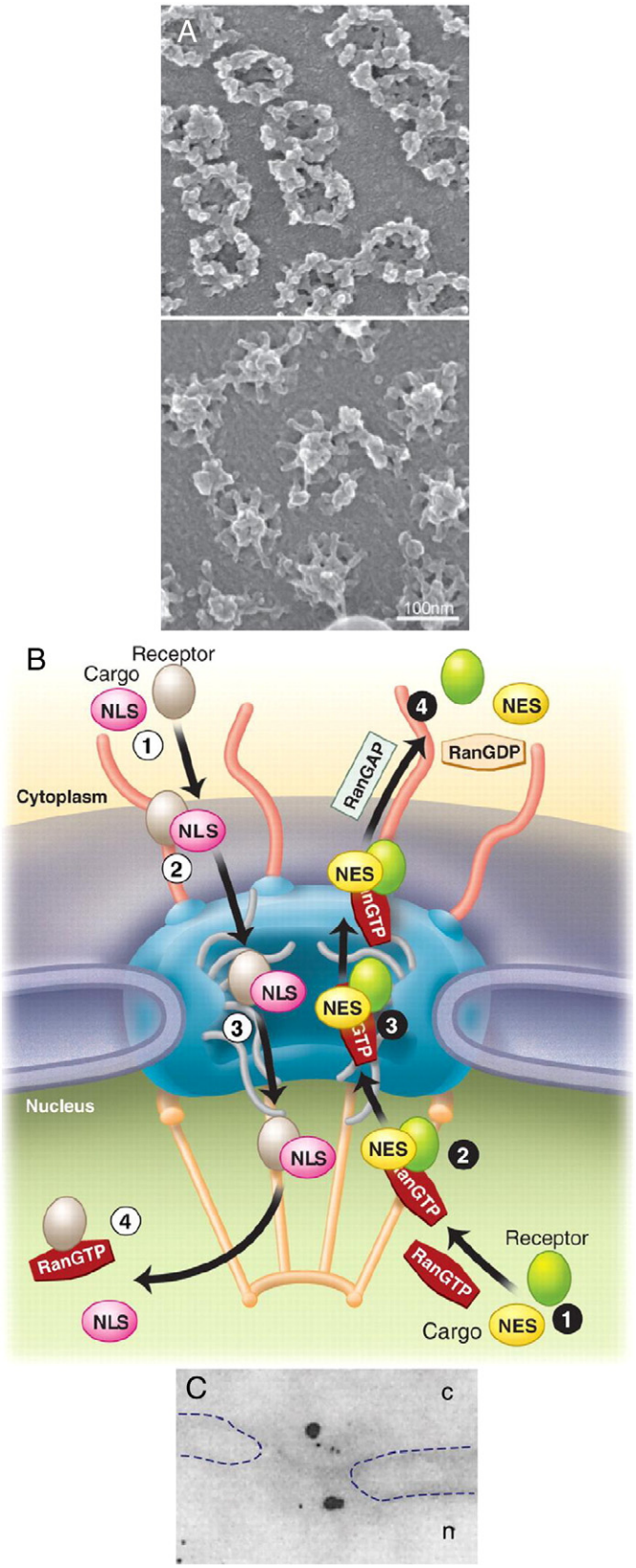

Fig. 6. (A) Scanning electron microscopic images of cytoplasmic (upper) and nucleoplasmic sides of nuclear envelope revealing the NPCs. (B) Schematic depiction of receptor-mediated transportation across the NPCs. (C) Immunoelectron microscopic image of gold nanoparticles' translocation between nucleus (n) and cytoplasm (c). Reproduced with permission from [207].

Recently, engineering dual (cellular and nucleus)-targeted nanoparticles with the goal of enhancing cellular uptake and localization to the nuclear compartment has emerged as an interesting challenge. For instance, Hoang et al. [210] developed PEG-PLA block copolymer micelles (~30 nm) labeled with Indium-111 (Auger electron emitter, ^{111}In), conjugated with trastuzumab fab (HER2-specific antibody) and NLS (CGYGPKKKRKVGG) with a further option of loading with the antimetabolite methotrexate. Based on the *in vitro* subcellular fractionation using cells expressing high levels of HER2 (MDA-MB-231, MDA-MB-361, and SK-BR-3 cell lines), the NLS block copolymer micelles showed higher accumulation in the nuclei, and the dual targeting platform exhibited an improved antiproliferative effect. Yu et al. [211] also employed the dual-targeted strategy and fabricated DOX-loaded folic acid and NLS (Ac-CGYGPKKKRKVGG)-functionalized chitosan (modified with cholesterol) micelles (~250 nm). The dual targeting led to a higher cellular uptake (KB cells) because of the folic acid and increased nucleus localization due to the NLS, and the highest tumor suppression (KB tumor xenograft models, BALB/c nude mice) when compared to the nontargeted and singly targeted DOX loaded micelles.

As presumed in some of the studies above, nuclear access by these large nanoparticles is possible only if they undergo a degradation or micellar disassembly process after delivery into the cytoplasm. Keeping in mind the NPC channel size (~39 nm), a comprehensive evaluation of the size range of nanoparticles with different physiochemical properties that can translocate across the NPC channels is necessary for designing optimum nuclear-targeted nanoparticles.

5. Outlook

The success of nanoparticle-based carrier systems in human trials for the targeted delivery of therapeutic agents reflects the progress of nanomedicines towards the clinic. For the development of more potent nanomedicines, an in-depth understanding of cellular uptake mechanisms is of paramount importance. Nanoparticles enter the cells via a combination of different internalization routes. Depending on the size, shape and surface charge of the nanoparticles, a particular cellular internalization route may be preferred over others. Since cellular internalization routes determine the fate and intracellular localization of nanoparticles, the development of reliable strategies to control or at least influence the nanoparticle cellular internalization route can affect therapeutic outcome. Along the same lines, a systematic investigation of abnormally high nutrient uptake during tumor progression as a route for delivery of drug-loaded nanoparticles may offer a unique opportunity for nanoparticle-based cancer therapy. With the new goals of subcellular organelle-level targeting, the field of nanomedicine is now moving to a higher level of complexity. Though the task is challenging, there are promising results highlighting the potential advances that can be expected from organelle-level targeting. Endosomes are organelles with slightly acidic pH that are encountered by all nanoparticles. Polyamines have been generally employed to trigger the release of nanoparticles from endosomes via the proton sponge effect, though questions have been raised regarding the poor biocompatibility or toxicity of polyamines because of their high pKa (~9). The development of low-pKa materials capable of triggering endosomal release may gain the clinical advantage of the proton sponge effect. The concept of subcellular-targeted nanoparticles is in its infancy, and few strategies have so far been reported for ER, mitochondria, and nucleus targeting. More detailed investigations are needed to assess the impact and relevance of subcellular targeting for future clinical applications. For subcellular targeting, it is desirable that the design of the nanoparticles retain the non-immunogenic "stealth" character with high systemic circulation combined with the ability to overcome biological barriers and target-site specificity. Though it presents a formidable challenge, nanoparticles capable of sequential multistage targeting can be an interesting strategy and the coming years should witness the success of this new paradigm in nanomedicine.

Acknowledgments

This work was supported by the National Cancer Institute (NCI) (grant U54-CA151884), the National Heart, Lung, and Blood Institute (NHLBI) Program of Excellence in Nanotechnology (PEN) (contract #HHSN268201000045C), the National Institute of Biomedical Imaging and Bioengineering (NIBIB) R01 grant (EB015419-01), the National Research Foundation of Korea (K1A1A2048701), Movember Challenge Award from Prostate Cancer Foundation, and the David Koch–Prostate Cancer Foundation Award in Nanotherapeutics. J.S. thanks the support from NCI R00CA160350 and Prostate Cancer Foundation Young Investigator Award. C.V. acknowledges the support from Center for the Development of Nanoscience and Nanotechnology (Grant FB0807) and the Postdoctoral Program of Becas-Chile/CONICYT.

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