



Published in final edited form as:

J Control Release. 2014 September 28; 190: 485–499. doi:10.1016/j.jconrel.2014.06.038.

Insight into nanoparticle cellular uptake and intracellular targeting

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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 an efficient cellular uptake through the plasma membrane, and the development of nanomedicines for precise delivery to subcellular compartments remain formidable challenges. The cellular internalization routes have a determining effect on 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 even resulted in a remarkable development towards effective oral delivery of nanomedicines that can overcome the intestinal epithelial cellular barrier. A detailed overview of the recent developments towards subcellular targeting that is emerging as a platform for the next generation organelle specific nanomedicines is also provided. Each section of the review includes prospect, potential, and concrete expectations from the field of targeted nanomedicines and strategies to meet those expectations.

Keywords

Nanomedicine; nanoparticle cellular uptake; non-targeted and targeted nanoparticles; intracellular distribution; subcellular targeting

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The rest of the authors declare no conflict of interest.

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1. Introduction

The multidisciplinary and integrative research efforts in the field of nanomedicine have led to the development of a variety of nanoparticle-based carrier systems potentially suitable for site specific delivery of diagnostic and therapeutic agents [1]. The original foundation for the recent revolutionary leaps taken by nanomaterials for biomedical application is considered to be laid in 1960 through the famous lecture of R. Feynman “*There is plenty of room at the bottom*” [2]. However; the work of Paul Ehrlich, who coined the visionary idea of “*magic bullets*”, related to the cell-specific diagnostics and cell-targeted therapies is also of seminal importance [3,4]. The field of nanomedicine has established its ability to overcome the poor solubility, non-specific cytotoxicity, poor bioavailability, and less than optimum pharmacokinetic 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 in the development of nanomedicines [5–8]. The whole idea of nanomedicines, on one hand, has been augmented 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. While, on the other hand, 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 development and to realize bench-to-bedside translation. The recent success of nanomedicines stems from some key developments of multidisciplinary nature. The non-fouling nature of hydrophilic materials such as polyethylene glycol (PEG) and polycarboxybetaine (PCB) [9,10] against biological materials, and observation of the enhanced permeation and retention (EPR) effect are two such examples [11]. The development of hydrophilic polymers 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] associated with the tumor because of the abnormal and leaky microvasculature has emerged as the foundation for the passively targeted first generation of nanomedicines preferentially accumulating in tumor tissue [15, 16]. The combination of hydrophilic polymers induced long systemic circulation 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 the proximity to a diseased tissue or inside the cells after internalization. The EPR effect is a scientific observation that results from many complex biological processes reviewed in ref 11, however; the therapeutic outcome based on exploiting the EPR effect can be inconsistent due mainly to the heterogeneity associated with the tumor tissues. Recently, the specific affinity of receptors to certain ligand molecules has led to the second generation of nanomedicines that 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 at the surface of nanocarriers that results 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 have 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 on one hand highlights the importance of a better understanding of cellular uptake mechanisms while on the other hand it has fueled recent research activities focused on the development of nanocarriers capable of subcellular and organelle level targeting, referred to as third generation of nanomedicines [21]. After giving an account of endocytic pathways relevant for the non-targeted and ligand conjugated targeted nanoparticles, we herein provide a comprehensive review on the 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 organ, tissue and cells [22] has been the primary focus of nanoparticles based therapeutic strategies. The drug loaded nanoparticles have to overcome a number of transport barriers to reach the specific cells [23]. Particularly for intracellular targeting, an efficient translocation of nanoparticles across the plasma membrane barrier is a prerequisite. Plasma membrane provides an independent environment to develop the normal function of the different types of cells and presents high complexity. Plasma membrane also has a critical function in the cellular adhesion, communication, and division, and endocytosis plays a crucial role in the regulation of these critical functions of plasma membrane. The process of 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 phenomenon opposite to endocytosis, named as exocytosis, is the fusion of inner vesicles with the plasma membrane as a means to transporting molecules either to plasma membrane or to extracellular space [24]. The 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 endocytosis pathways employed by the cells. The phagocytosis route is mainly used by dendritic cells, neutrophils and macrophages [26]. The pinocytosis route is present in all types of cells and can be further subdivided as clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin/caveolae-independent endocytosis, and macropinocytosis. An efficient uptake of nanoparticles is important for an effective intracellular drug delivery; we believe that an understanding of the biological pathways for cellular internalization of nutrients and solutes can facilitate the development of nanoparticles that can perform precise intracellular targeting with enhanced therapeutic outcomes.

2.1 Phagocytosis

The phagocytosis is an endocytosis process exhibited by several types of cells, including epithelial cells, fibroblast, 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, the phagocytosis is used

to engulf the disabled particles, senescent cells, and infectious microorganisms (bacteria and virus) as a response of innate and adaptive immunity [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]. The process of 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 the opsonization process 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 (Fc γ RI, Fc γ RIIA and Fc γ RIIA), the complement receptors (CR1, CR3 and CR4), and the α 5 β 1 integrin [30]. A great deal of scientific efforts has been focused on controlling the nanoparticles 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 nanoparticles 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] have revealed that the particle geometry can help in modulating their cellular internalization via phagocytosis. Different local particle shapes at the point of cell attachment generates 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. Six distinct shapes of nanoparticles investigated the Mitragotri and coworkers include spheres (radius 1.0–12.5 μ m), oblate ellipsoids (major axis 4 μ m, aspect ratio 4), prolate ellipsoids (major axis 2–6 μ m, aspect ratio 1.3–3), elliptical disks (major axis 3–14 μ m, aspect ratio 2–4, thickness 400–1000 nm), rectangular disks (major axis 4–8 μ m, aspect ratio 1.5–4.5), and UFOs (sphere radius 1.5 μ m, ring radius 4 μ m). The authors demonstrated that elongated particles with higher aspect ratio are less prone to the phagocytosis. Geng et al. [33] have also reported a similar finding. Interestingly, a higher aspect ratio has also recently been implicated with a preferential localization into endosomes and lysosomes [34], hence, care should be taken while exploiting the shape of particles for modulating the 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 the eukaryotic cells. CME is a complex pathway that involves intercellular signaling, membrane recycling, and uptake of nutrients [35]. The 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. Majority of the receptor mediated cellular uptake of nanoparticles occur through CME. Particular examples are provided in the later sections dealing with the ligand targeted nanoparticles. In 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. The cationic nanoparticles of ~100 nm size derived from the polylactide-*co*-polyethylene glycol (PLA-PEG) have been found to internalize exclusively via CME [47]. The poly(L-lysine), which is a cationic polymer, functionalized at the surface of poly(lactide-*co*-glycolide) (PLGA) nanoparticles have also been found to significantly enhance the cellular uptake via CME [48]. Another study reported mesoporous silica nanoparticles (~110 nm) labeled with fluorescein isothiocyanate showing efficient internalization 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 cell activity has resulted in many studies concluding the CME as a main route of internalization for nanoparticles of size ~100 nm. The positively charged nanoparticles of ~100 nm diameter have been shown to internalize predominantly through CME, which can be a result of increased interaction between the positively charged nanoparticles and negatively charged cell surface that enhances the nanoparticles internalization via CME simply because the CME is the most probable internalization route for nanoparticles of ~100 nm diameter. To the best of our knowledge, there is no systematic study on ~100 nm size nanoparticles investigating the effect of nanoparticles shape on the cellular internalization route. All the reported literature in this size context deals with the nanoparticles of spherical shape.

2.2.2. Clathrin-independent endocytosis—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 in the plasma membrane repair, cellular polarization, cellular spreading, and modulation of intercellular signaling [50]. CIE does not require the presence of coat proteins for the vesicle formation and internalization; however, the actin and actin-associated proteins are important players for the vesicle formation during CIE [51]. The CIE involves different subtypes of pathways that include the participation of proteins such as Arf-6, RhoA, and Cdc42 [52]. Studies have shown that Arf-6-dependent CIE participates in the endocytosis of the major histocompatibility complex (MHC) class I [53], the β -integrins [54], the glucose transporter GLUT1, and other proteins that are involved in amino acid uptake and cell-extracellular matrix interactions [55]. In addition, RhoA and Cdc42 endocytosis are dependent on the 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, the 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]. The cargos entering the cell through CIE are usually

delivered to the early endosomes, followed by the transfer to late endosomes and lysosomes. In addition, the 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 oligomers nanoparticles (SBTCO) for the delivery of DNA [60], and for cowpea mosaic virus (CPMV), which has been extensively studied in the last 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 new mechanism for the uptake of nanoparticles that was described as a type of macropinocytosis. This new mechanism was found to be dependent on the actin filaments and dynamin, and was designated as *excavator shovel* like mechanism [62]. In another study, Garaiova et al. [63] showed that the nanoparticles derived from trisaccharide-substituted chitosan oligomers (SBTCO) generated a higher uptake and better transfection efficacy than the nanoparticles prepared from a linear chitosan (LCO). SBTCO were primarily taken up by the cells via CIE, and successfully escaped from the endocytic vesicles. In contrast, LCO suspension in the cell culture medium resulted in the nanoparticles aggregation and a relatively lower extent of cellular internalization was observed when compared to the SBTCO nanoparticles.

2.2.3. Caveolae—Caveolae are the 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 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 case of the endothelial cells in blood capillaries [66]. Initial studies have revealed the caveolin (CAV1, CAV2 and CAV3) as the main protein constituent of caveolae with an estimate of about 140–150 of CAV1 protein molecules per caveolae [67]. Cavins, the coat proteins (cavin 1–4), are known to work together with caveolins to regulate the formation of caveolae, and also potentially participate in the signals that regulate the caveolae fate [68]. The intracellular destinations of caveolae have been a subject of controversy for many years. Nevertheless, it has emerged that in endothelial cells caveolae are able to perform transendothelial transport, which may be utilized for the release of nanoparticles in the subendothelial tissues [69]. The material that is endocytosed via caveolin-mediated pathway is initially localized into caveosomes. The neutral pH of caveosomes can be considered as a means to avoid the hydrolytic environment of lysosomes. The sorting of caveosomes cargo to the Golgi apparatus and endoplasmic reticulum may also be exploited for the targeted delivery of theranostic agents to these subcellular compartments. The negative surface charge has been found to trigger the 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 DNA conjugated poly(amido amine) (PAMAM) dendrimer, exhibiting significant accumulation of carrier in the brain of mice after intravenously 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 that involved an interaction with GABA_B receptor [70]. Interestingly, previous studies about the

mechanism of rabies virus glycoprotein crossing the blood brain barrier (BBB) and 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 delivery of drugs to 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 the antigen presentation in major histocompatibility complex II (MHCII) [76, 77]. Unlike the receptor-mediated endocytosis and phagocytosis, the activation of macropinocytosis is not regulated by the direct action of a receptor or the cargo molecules. In this case, the activation of tyrosine kinase receptor such as the epidermal growth factor and the platelet-derived growth factor receptor leads to an increment of the actin polymerization, actin-mediated ruffling, and macropinosome formation [78]. Interestingly, macropinosomes share some proteins (Cdc42, Arf6 and Rab5) with others endocytosis processes, suggesting a relationship between the mechanisms of macropinosomes biogenesis and other endocytosis routes [79, 80]. The macropinosomes are sensitive to cytoplasmic pH and undergo acidification and fusion events [81]. In macrophages, the macropinosomes present a fate similar to endosomes, and during their maturation gain and loss markers that are typical for early and late endosome before their fusion with lysosomes [82]. Micron size particles are generally known to internalize the cells via macropinocytosis [83], however; most of the literature reports highlight that the nanoparticles undergo cellular internalization via more than one endocytic pathway. Recently, Zhang et al. [84] reported lapatinib loaded nanoparticles formulated with a core of albumin and a lipid corona formed by the egg yolk lecithin. The nanoparticles exhibited ~62 nm and a zeta potential of 22.80 mV and were demonstrated to internalize the 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

The essential nutrients, such as lipids, free forms of fat-soluble vitamins and carotenoids, transport across the plasma membrane by simple diffusion [85]. The transport of some nutrients such as glucose or lipoproteins is actively controlled by the endocytosis 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 case of cancer progression concomitantly triggers a higher rate of nutrients uptake. Several studies support that the actively proliferating cells could adapt their endocytosis mechanisms to meet the enhanced nutrients requirement. Recently, a study on human melanoma cells revealed a significantly increased expression of the aminoacid transporter for leucine (LAT1) and glutamine (ASCT2) [88]. Another study on pancreatic tumor xenograft model suggests that the macropinocytosis is an important route for nutrient uptake in tumor cells [89]. This study reveals the enhanced rate of albumin uptake for meeting a higher glutamine demand of cancer cells (MIA PaCa-2 cells) as a functional contribution of the macropinocytosis stimulated by the oncogenic Ras proteins. These hyperactive endocytic

mechanisms have not been explored in context of nanomedicine and present a potential strategy for enhancing the uptake of therapeutic nanoparticles in cancer cells. For any future development in this context, an indepth understanding of the differential physiopathological processes associated with the diseased and normal cells is a prerequisite to enhance the relevance of differential nutrient uptake phenomena as a gateway for cellular internalization of nanomedicines.

Keeping in view the fact that size, geometry, surface charge and mechanical properties of nanoparticles can influence the cellular uptake efficiency, several studies have been focused on understanding the optimal properties of nanoparticles to accomplish an effective cellular uptake and consequently an enhanced pharmacological effect. These details are comprehensively covered in the following excellent review articles [11,90,91]. In addition to tuning the physical characteristics, receptor mediated cellular internalization has also been extensively employed for achieving an enhanced 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 receptors overexpressed on the surface of target disease cells have been widely explored to improve the cellular uptake of nanoparticles as well as to minimize the off-site toxicity concerns. Currently, several receptors are known for active disease cell targeting; the representative examples 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 at the surface of nanoparticles in order 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 this section, the recent developments related to the targeting strategies using ligand-conjugated nanoparticles will be addressed highlighting the receptors (FR, TfR, EGFR, PMSA, Integrin, FcRn) 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 frequent tumor types (e.g., ovarian, lung, brain, and colorectal cancer) [93]. FR is a glycoprotein with molecular weights of 38–44 kDa and exists in isoforms FR- α and FR- β . The 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 kidneys [94]. In contrast, the 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, the FR- β can prove to be an important target. Folic acid (441 Da) shows a high affinity ($K_D = 10^{-9}$ M) to the FR, which

allows the selective delivery of folate-conjugated nanocarriers to the FR-expressing disease cells [97]. The use of small molecules like folic acid ligand compared with the peptides and antibodies as targeting ligands can provide several benefits. The main advantages include an easy scale-up for clinical applications, facile chemical modification, no toxicity and immune reactions due to its function as a vitamin, and the 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]. The PEGylated liposomal doxorubicin (DOX) containing folate-PEG-distearoyl-phosphatidyl-ethanolamine showed higher therapeutic activity than that of non-targeted one 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 the FR [99]. Jiang et al. developed folate-conjugated human serum albumin nanoparticle loaded with docetaxel and investigated the antitumor activity of the nanoparticles in human hepatoma cell line and in an *in vivo* model [100]. The folate-conjugated nanoparticles were found to show superior antitumor activity based on *in vivo* inhibition ratios. Chemotherapeutic drugs loaded folate functionalized polymer derived nanoparticles have been reported to exhibit significantly higher *in vivo* efficacy as compared to the non-targeted nanoparticle therapeutics as evaluated in an ovarian peritoneal metastasis model [101]. High affinity of folate moieties is also of interest for use in the fields of immunotherapy as well as leukemia cells. Lu et al. were able to demonstrate that a treatment with folate-conjugated hapten resulted in more immunogenic tumor cells and finally the enhanced anticancer immune reaction against hapten-treated tumor cells [102]. For targeting the acute myelogenous leukemia (AML) blast cells with overexpressed FR- β (70%), the folate conjugated liposomal DOX resulted in a stronger inhibition of colony formation than that of the non-targeted analog in MV4-11 (human acute myelocytic leukemia) and K562 (human erythromyeloblastoid leukemia) cells and AML patient cells as well [103]. These studies suggest the efficacy and the potency of folate targeting in pre-clinical as well as clinical application.

3.2. Transferrin receptor (TfR) targeting

The transferrin receptor (TfR), which is a cell membrane glycoprotein with a homodimer of two identical transmembrane subunits, can mediate 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, especially 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 the cellular uptake of iron from transferrin (~80 kDa) and cell growth [107], and TfR is probably expressed on all cells and the level of TfR expression varies among different cells. A high expression of TfR is known for immature erythroid, placental tissue, rapidly dividing cells, and its expression is about 100-fold higher in cancer cells than the regular expression of normal cells [108], which makes TfR as 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. The

examples of TfR targeted nanomedicines that are at various stages of clinical trials include CALAA-01 (a four component nanomedicine in phase I clinical trial constituted by Tf-functionalized PEG, Tf and adamantane functionalized PEG, SiRNA and a cyclodextrin bearing polymer), MBP-426 (liposome based oxaliplatin loaded Tf conjugated nanomedicine in Phase II clinical trial), SGT-53 (intracellular delivery of p53 plasmid DNA using TfRscFv conjugated liposome based nanomedicine in Phase Ib clinical trial), and SGT-94 (RB94 plasmid DNA delivery using TfRscFv conjugated liposome in Phase I clinical trial) [109]. These successful examples have triggered substantial interest in the further development of TfR targeted nanomedicine. Choi et al. reported that PEGylated gold nanoparticles with different amounts of human transferrin for active targeting leading to an efficient nanoparticle internalization into TfR over expressing cancer cells (Neuro2A cells) as well as in the tumor site in Neuro2A tumor-bearing mice, suggesting greater intracellular delivery of therapeutic agents to the target cancer cells [110]. For effective treatment of brain glioma cells, the 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 resulted in an enhanced anti-glioma activity compared to the non-targeted nanoparticles [111]. The transferrin receptor mediated internalization of the anticancer drugs into the brain tumor has been associated with the highly overexpressed TfR in brain capillary endothelium and glioma cells. In addition, Hong et al. showed that the endocytosis process of Tf-PEG-niosomes in KB cells could be inhibited by low temperature and by the free Tf, indicating the high specificity towards TfR [112]. As expected, the hydroxycamptothecin (HCPT)-loaded Tf-PEG-niosomes resulted in the strongest cytotoxicity to three carcinomatous cell lines (KB, K562, and S180 cells) and significantly stronger inhibition (71%) of tumor growth in S180 tumor-bearing mice compared to the 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 report from Salvati et al. [113] is of worth mentioning that reports disappearance of targeting ability of Tf functionalized nanoparticles in the biological media. The protein constituents of the biological media were implicated for neutralization of TfR targeting of Tf ligands that were covalently localized at the surface of nanoparticles through a PEG linker. This finding is of paramount importance necessitating the evaluation of any potential targeting nanomedicine in the biological medium rather than in buffer saline. It further highlights the importance of evaluation of optimum ligand density to achieve a balance between desired systemic circulation and 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 including HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [114,115]. The 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]. The small molecules and monoclonal antibodies have been used as EGFR-targeting ligands; for examples, epidermal growth factor (EGF), transforming growth factor- α (TGF- α), heparin binding EGF-like growth factor, epigen, betacellulin, 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 the cellular signaling.

Anti-EGFR ILs-DOX (Fab' fragments of anti-EGFR antibody ceuximab conjugated DOX loaded immunoliposomes targeted to EGFR in Phase I clinical trial) represents a notable example of EGFR targeted nanomedicines [109]. In a recent attempt of targeting HER1, Shevtsov et al. developed the superparamagnetic iron oxide nanoparticles conjugated with recombinant human epidermal growth factor (SPION-EGF) for the enhanced magnetic resonance imaging of malignant brain tumors and demonstrated more efficient tumor imaging than that of 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 effect in A549 cells and tumor-bearing mice model (high EGFR expression) compared to HFL1 cells (low EGFR expression) [119]. For HER2 (14–91% of patients with breast cancer) targeting, 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 the DOX-loaded PEG-PLGA-Au half-shell nanoparticles with Herceptin resulted in higher accumulation in tumor sites of SK-BR-3 (breast cancer cell line with high HER2 expression) bearing mice by receptor-mediated endocytosis and finally stronger therapeutic efficacy based on chemotherapy and hyperthermia compared to non-targeted nanoparticles [121]. MM302 (HER2 targeted scFv antibody fragment conjugated DOX loaded liposome) represents an example of HER2 targeted nanomedicine in Phase I clinical trial [109]. Currently, HER3, which is the only member of EGFR family lacking intrinsic tyrosine kinase activity, has gained significant interest for targeting of EGFR [122]. The fully humanized HER3 antibody U3-1287 (AMG 888) showed a reduced growth of cancer cells and improved tumor suppression in xenografted human HNSCC FaDu model. Therefore, based on these research results, the cellular targeting strategies by receptor-mediated endocytosis will potentially impact in clinical application to treat various diseases, specifically cancers.

3.4. Prostate-specific membrane antigen (PSMA) targeting

A type 2 integral membrane glycoprotein PSMA is a valid cancer target overexpressed on the surface of prostate carcinomas and the neovasculature of majority of the solid tumors [123,124]. In this regard, a notable example is represented by the BIND-014, docetaxel leaded polymer (PLA-PEG and PLGA-PEG) based nanomedicine in Phase II clinical trial, 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]. A combinatorial approach, screening about 100 formations, was employed to achieve the effective nanomedicine attributes including particle size, targeting ligand density, surface hydrophilicity, drug loading, systemic circulation and drug release properties (Fig. 2). This thorough optimization resulted in a highly effective PSMA-positive prostate cancer targeted nanomedicine with remarkable results of tumor shrinkage in human at doses below the typical clinical doses of solvent based docetaxel formulation (Fig. 2).

3.5. Integrins targeting

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

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

Nanomedicine administration is currently limited to the parenteral routes, however; for convenience and patient compliance oral route of administration is of particular interest, especially against the diseases that require frequent administration [133]. Oral route of administration suffers from a 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, highlighting a gateway to overcome intestinal epithelium cellular barrier [135]. In adults the FcRn receptor expression level in apical region of epithelial cells of small intestine is equivalent to the neonatal expression. Interestingly, the affinity between Fc region of IgG and FcRn is pH dependent, high binding affinity at acidic pH (<6.5) as compared to the physiological pH (~7.4). Capitalizing on these facts, Pridgen et al. [136] have reported a ground breaking proof of concept study demonstrating 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) employed in this study exhibited transepithelial transport both *in vitro* and *in vivo*. Oral administration of fluorescently labeled NP-Fc in fasted wild-type mice resulted in observation of fluorescence revealing the ability of Fc functionalized nanoparticles to overcome the intestinal epithelial barrier and entering into the lamina propria (Fig. 3). The efficiency of absorption for targeted nanoparticles was 11.5 times higher than the nontargeted particles. The particles were also observed to localize in other organs, which shows that they were able to enter systemic circulation (Fig. 3). Furthermore, oral administration of insulin loaded NP-Fc in fasted wild-type mice resulted in a hypoglycemic response. A control experiment with the FcRn knockout mice did not result in a hypoglycemic response, which established that the superior outcome is because of FcRn targeting. Although this novel avenue is still at its infancy but the initial results are

promising enough to expect an impact on future development of nanomedicines that can be administered orally.

4. Intracellular trafficking and subcellular targeting

4.1 From endosomes/lysosomes to cytoplasm

Ligand conjugated nanoparticles with an ability of specific tissue or cellular level targeting has already been successfully demonstrated, and this novel paradigm goes in parallel with the EPR effect for achieving an increased intratumoral concentration of the cytotoxic anticancer drugs. Many interesting therapeutic targets are localized in the intracellular compartments. This has triggered increasing efforts focused on developing sophisticated nanoparticles designs with an ability to precisely navigate across the physiological barriers and selectively deliver the therapeutic and diagnostic agents to the 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) that has been exploited to trigger the release of pH responsive nanoparticles into the cytosol. Polyamines based carrier systems can efficiently impart neutralization effect after being protonated under the acidic conditions of endosome. This neutralization leads to an increase in endosomal pH that triggers the ionic transport into the lumen of endosome resulting in swelling and possible release of endocytosed nanoparticles (proton sponge effect) [138]. The choice of material to induce proton sponge effect has been limited to polyamines, however; these materials suffer from the poor biocompatibility and high toxicity inherent with the high pKa (~9) of amine groups that can induce membrane lysis at acidic as well as physiological pH. The protection of amine groups by employing acid sensitive protecting groups that cleave and expose the amino groups only under the endosomal pH to trigger the endosomal release, and incorporation of low pKa heterocycles as pendant groups have been reported to reduce the toxicity of the polymer system [139]. A timely response to the pH is very crucial as failing to respond efficiently can delay the release resulting in either transfer of endocytosed nanoparticles to the hydrolytic enzymes containing lysosomes for degradation or directed to the plasma membrane via recycling route.

The intracellular spatiotemporally controlled trafficking and the fate of endocytosed cargo are regulated by RAB proteins (one of the five major subfamilies of the superfamily G-proteins) in combination with a number of effectors or regulatory molecules. For cancer progression, an increased activity of the RAB proteins has been implicated with an increased rate of growth factor receptors recycling to the plasma membrane. The therapeutic intervention to modulate RAB activity provides interesting and rather less explored potential targets [140,141].

A well regulated endocytosis and endosomal trafficking is of paramount importance as an improper functioning of endosomal trafficking has been linked to the neurodegenerative diseases (e.g., Alzheimer's disease, Huntington's disease, autism) [142–145]. Na-H exchangers (NHEs) is 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, recycling endosomes, and lysosomes, where

pH decreases to 5 [146,147]. With the maturity of endosome a 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 initiating and development of cancer [148]. Interestingly, all the malignant tumors have a reversed hydrogen gradient as compared to the normal tissues. The intracellular pH of the 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 under hostile extracellular environment. The dysfunctional pH control system 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. The NHE1 inhibitors are being employed as selective tumor drugs [34, 151, 152]. The field of nanomedicine is yet to contribute in this context; it is of paramount importance that these attributes should be considered while developing nanoparticles for cell membrane, endosome and lysosome targeting.

Lysosomes are considered as digesting or recycling components of the cell machinery because of the presence of cathepsins proteases. The resulting degradation products from the enzymatic activity on the cargo materials are released in the cytoplasm to meet the nutritional need of the cell. The tumor cells exhibit higher activity of lysosomal cathepsin and its release in the extracellular environment has been implicated with the promotion of tumor growth. The concept of lysosomotropic was introduced by Christian de Duve who defined the lysosomes with their hydrolytic enzymes as suicide bags [153]. Since then the destabilization of lysosomes via lysosomal membrane permeabilization (LMP) and release of its hydrolytic contents in the cytoplasm as a means of triggering cell death has been highlighted as potential targets for therapy. Multidrug resistance acquired by the cancer even at the early stage of its development makes the 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 the lysosomal membrane integrity was exploited to induce the 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 the normal cells [154–156].

In case the endosome or lysosome is not the final therapeutic target, it is necessary to devise a strategy for endosomal release and for the protection of administered drug from degradation under hostile environment of lysosomes. This has been achieved by encapsulating drugs into a variety of nanoparticle based carrier systems. These nanoparticles are not only required to protect the sensitive drug molecules from degradation but also needed to release the drug in a time dependent manner [157]. A 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 affect the release of a cargo

from endosome into the cytoplasm. These include the use of cell penetrating peptides, stimuli responsive polymers (endoosmolytic mechanism to induce the endosomal disruption and release by exploiting differential redox or pH environment) or use of 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 property 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 in contrast to its accumulation in the endosome and lysosome when cells were exposed to the free DND-26. DPA functional group is of particular interest because of its distinct pKa value (6.3) as compared to the pKa (7.4) of *N,N*-dimethylethylenediamine. A judicious choice of such functionalities can offer modulation of 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 to the side chain degradation resulting in the polymer charge reversal and consequently destabilization of nanoparticles and release of loaded lysozyme. Despite of several reports on stimuli responsive materials, there is hardly any nanoparticles platform at the clinical stage, which highlights a 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 for designing clinically relevant polymers.

Cytoplasm is home to a wide range of biological processes (signaling, metabolic and pathogenic) that have been demonstrated to be the therapeutic targets for many diseases. Access for nanomedicine to the cytoplasm has mainly been through the endosomes; however, some cell penetrating peptides (CPP) can enter into the cytoplasm by directly traversing the plasma membrane. The ability of CPP to directly translocate across the plasma membrane is greatly lost when conjugated with a cargo [163,137]. Some CPPs have, in fact, been employed as anticancer agent per se (e.g., Azurin against solid tumors under Phase I clinical, trial and XG-102 targeting c-Jun-N-terminal kinases under Phase II clinical trial). Co-administration of a CPP (iRGD) via systemic injection has resulted in improved therapeutic index of a variety of codelivered 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 α_v integrins, which are exclusively expressed on the tumor vessels endothelium. After preferentially homing to the tumors, iRGD is proteolytically transformed into the CRDGK/R that loses its affinity with the α_v integrins but acquires affinity to the neuropilin 1 (NRP-1) that triggers the tissue penetration by enhancing the vascular permeability. The whole process is tumor specific because of the α_v integrins binding specificity of iRGD. The iRGD assisted improved therapeutic index has been demonstrated only in the mouse tumor models, the efficacy of this platform is 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 represented by the retrograde trafficking pathway, which leads the endosomal cargo to the Golgi apparatus (GA) and endoplasmic reticulum (ER) [165]. ER and GA are responsible for calcium homeostasis, folding of membrane and secretory protein, and lipid biosynthesis. Retrograde trafficking pathway is involved in the recycling of certain receptors (e.g., mannose-6 phosphate receptor) and has been 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 ER [166,167]. An irregular ER function recognized as ER stress results in unfolded protein response (UPR) that leads to protein synthesis inhibition and refolding of proteins, and clearance of misfolded proteins. ER stress can lead to apoptosis that has been a cause of heart diseases (cardiac hypertrophy, degeneration of cardiomyocytes), liver diseases, neurodegenerative diseases, and diabetes [168,169]. UPR has also been proposed as 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, however; there are some notable examples available in the literature that may lay a foundation for such an endeavor in future. In an in vitro experiment PLGA (95 nm \pm 20) based nanocarriers are observed to accumulate predominantly in the Golgi apparatus in case of human bronchial epithelial (HBE) and opossum kidney (OK) renal tubule cells as revealed by the compartments labeling using immunofluorescence [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 a receptor mediated cellular internalization and subsequent associated with the GA via a retrograde route. The resolution of the analytical method used in this study was not able to conclude if 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 approaching the cells 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 the MDCK epithelial cells were predominantly transported to the lysosomes with some particles found localizing in the ER and Golgi complex emphasizing the critical importance of the internalization pathways [173]. The extent of nanoparticles 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 the cellular organelles comprising of two-membrane structure (inner and outer mitochondrial membranes) with the mitochondrial DNA enclosed in the inner membrane. The protein constituents of the mitochondria are comprised of

mitochondrial genome coded proteins or of nucleus origin. The mitochondrial diseases, therefore, originate from defective nuclear and mitochondrial genome. Mitochondrial dysfunction has been implicated for a number of diseases, including cancer, neurodegenerative and neuromuscular diseases, obesity, and diabetes, and has been recognized for hosting vital 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 a 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], fulfilling the prerequisite of a balance between the delocalized positive charge and the lipophilicity, has been shown to successfully cross this barrier and reach to 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 liposomes based nanocarrier platform has been demonstrated. In a series of attempts, TPP has been incorporated in the lipid bilayer membrane of liposomes by covalently conjugating 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 for a low octaarginine surface density liposomes were internalized via a clatherin mediated endocytosis followed by the transfer to lysosome for degradation [194]. Despite of the recent efforts showing some improvement, immune response and in vivo toxicity is a general concern associated with these targeting platforms [195,192]. Compared to the 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) nanoparticles based delivery system for targeted delivery of various mitochondria-acting drugs (Fig. 4). In order to optimize the particle size and surface charge for an optimum 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, authors could prepare nanoparticles of different sizes (ranging from ~80 nm to ~400 nm) with a constant surface charge (~ +30 mV) and nanoparticles of approximately same particle 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 a high accumulation in the mitochondria of human cervical cancer (HeLa) cell. Interestingly, the positively charged targeted nanoparticles were able to escape from early endosome and get localized in the mitochondria, whereas the negative charged non-targeted nanoparticles were found localized 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 the cargo release in the cytosol. In an attempt to demonstrate the non-aggregating behavior of positively charged nanoparticles that is generally induced by the negatively charged serum proteins, authors further reported stability of the nanoparticles by monitoring the change in the nanoparticles size in the cell culture media 10% (vol/vol) FBS in DMEM or 10% (vol/vol) FBS in H₂O for 7 days. The charged nanoparticles below 200 nm were found not to 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 prepared by encapsulation of mitochondria-acting drugs, including Isoniazid 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 exhibited marked improvement in 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. have 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 is needed to be evaluated for potential clinical applications.

4.4 Nucleus

Nucleus, a double lipid bilayer wrapped organelle hosting therapeutic targets (proteins, nuclear receptors, and DNA) of a variety of diseases, has been a focus of targeted drug and DNA (DNA as a drug for gene therapy) delivery. For 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-cargo complex. 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 stands for polymers with 10 and 15 L-lysine repeat units, Fig. 5) [198]. pHK10 copolymer interacted with the plasmid DNA resulting in the nanoparticles with lower aspect ratio (width ~25 nm and length ~74 nm) when compared to the nanoparticles (width ~18 nm and length ~102 nm) formed by the copolymer pHK15. In this study, a higher aspect ratio was implicated with the 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 especially of interest as this cell internalization pathway results in delivering 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 GA and ER that resides in close proximity to nucleus, and have been proposed to provide nanomedicines with a safe route to nucleus [199–201]. Certain functionalities such

as L-arginin [202] and saccharide [203] moieties as constituents of carrier polymer systems have been reported to sort the polyplexes to GA and ER. Particularly 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 may provide a convenient way of caveolae-mediated nucleus targeting. In the similar vein, β -cholanolic acid based hydrophobically modified glycol chitosan polymer nanoparticles (~359 nm) were found within the cytoplasm of the HeLa cells just after two minutes of incubation with almost no localization in the lysosomes. About 20% of the particles were found localized in the lysosomes after 60 minutes 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. In case, the drug resistance machinery of the cell has been activated the drug molecules have an even meager chance to reach to the target. However, establishing a strategy to navigating through the cytosol and reaching precisely at the nuclear target 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 the specific targets.

After surmounting the plasma membrane barrier, nuclear envelope presents another barrier for the nuclear delivery. The 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 (Nup), determine their assembly, structural, and functional aspects. Nature employs two modes of transportation for trafficking across the NPCs, passive and active. The small ions and macromolecules (~9 nm) are transported across the nuclear envelop via passive diffusion through the NPCs while macromolecules larger than 40kDa (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). FG-Nups constituent of NPCs, contain phenylalanine-glycine (FG) domains, 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 for translocation of cargo across the NPC [207].

The conjugation of nuclear localization signal (NLS) to the 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 dots conjugated PLGA nanoparticles (~168 nm) were observed to be localized in the nucleus of HeLa cells. In the similar vein, NLS functionalized DOX loaded PLGA nanoparticles (~226 nm) were shown to deliver great amount of the drug to the nucleus (MCF7 cells) when compared to the free drug of PLGA nanoparticles lacking an NLS. The conjugation with NLS further enhanced the cell cycle (G2/M phase) blocking capacity and induced higher extent of apoptosis [209].

Recently, engineering dual (cellular and nucleus) targeted nanoparticles with a 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 cells 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 highest tumor suppression (KB tumor xenograft models, BALB/c nude mice) when compared to the nontargeted or singly targeted DOX loaded micelles.

As presumed in some of the studies above, the nuclear access to these large nanoparticles can only be possible if they undergo a degradation or micellar disassembly process after delivery in the cytoplasm. Keeping in view 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 an optimum nuclear targeting nanoparticles.

5. Outlook

The success of nanoparticles based carrier systems in human trials for the targeted delivery of therapeutic agents reflects an active 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 different internalization routes. Depending on the size, shape and surface charge of the nanoparticles, a particular cellular internalization route may be preferred on the others. The fact that the cellular internalization routes determine the fate and intracellular localization of nanoparticles emphasizes that the development of reliable strategies to control or at least influence the nanoparticle cellular internalization route can hugely augment the therapeutic outcome. In the same vein, a systematic investigation of abnormally high rate of nutrient uptake during the tumor progression as a route for delivery of drugs loaded nanoparticles may offer a unique opportunity for nanoparticles based cancer therapy. With the new goals set for the subcellular organelle level targeting, the field of nanomedicine is now moving to the next level of complexity. The task is challenging, however; there are notable reports showing promising results highlighting the potential improvements that can be expected from organelle level targeting. Endosomes are the organelles with slightly acidic pH that are encountered by all the nanoparticles. Polyamines have been generally employed to trigger the release of nanoparticles from the endosomes via proton sponge effect, however; questions have been raised regarding the poor biocompatibility of toxicity of polyamines because of their high pKa (~9). The development of low pKa materials capable of triggering the endosomal release may result in the clinical advantage of proton sponge effect. The concept of subcellular targeted nanoparticles is at its infancy and only a limited number of 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 their future clinical applications. For subcellular targeting, it is desirable that the design of the nanoparticles still present the non-immunogenic stealth character with high systemic circulation combined with the ability of overcoming biological barriers and target site specificity. It is a formidable challenge, however; nanoparticles capable of exhibiting a sequential multistage stage targeting can be an interesting strategy and coming years should witness the success of this new paradigm of nanomedicines.

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 #HHSN26820100045C), the National Institute of Biomedical Imaging and Bioengineering (NIBIB) R01 grant (EB015419-01) and the David Koch-Prostate Cancer Foundation Award in Nanotherapeutics. Dr. Farokhzad declares financial interests in BIND Therapeutics, Selecta Biosciences and Blend Therapeutics; three biotechnology companies developing nanoparticle technologies for medical applications. 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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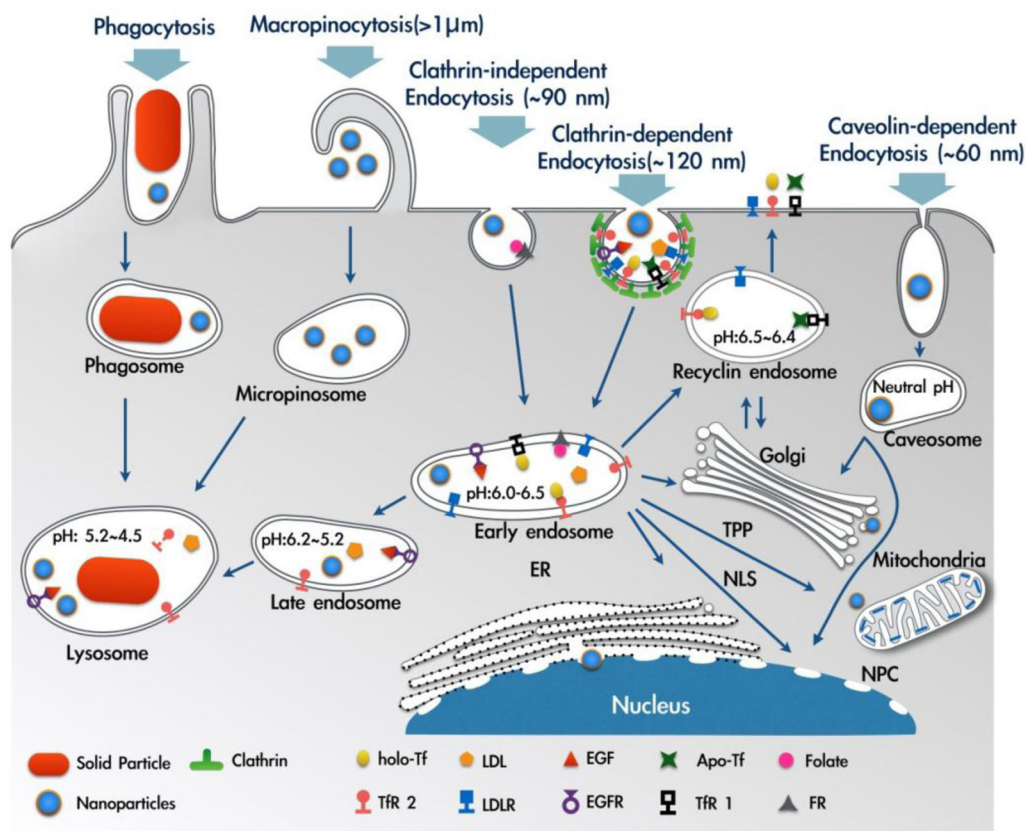


Fig. 1. Illustration of internalization pathways discussed in this article (phagocytosis, macropinocytosis, clathrin-dependent endocytosis, clathrin-independent endocytosis, and caveolin-dependent endocytosis). The fate of internalized cargo and localization to subcellular compartments are also depicted.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

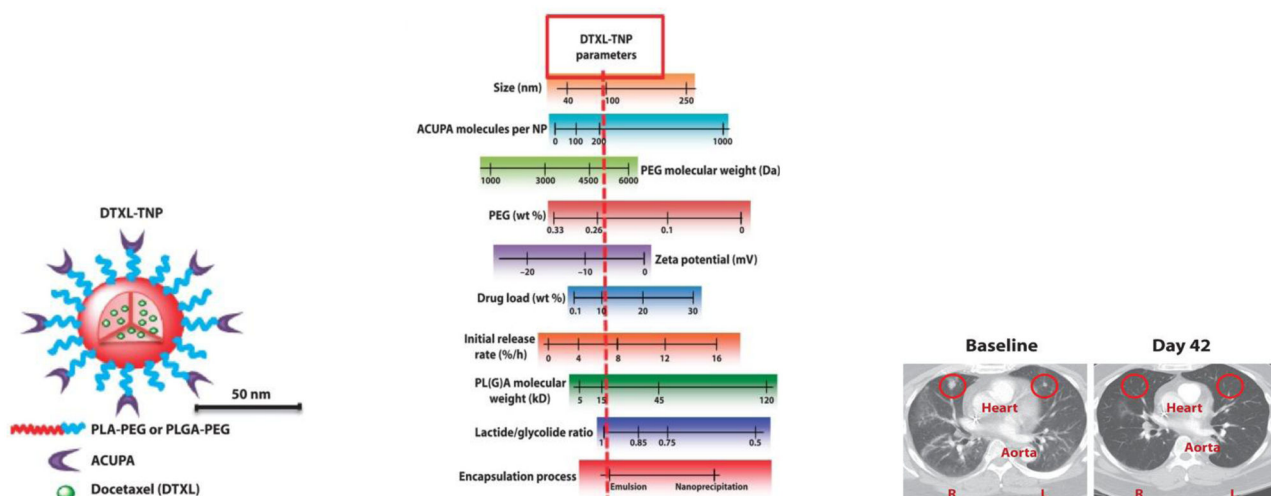


Fig. 2. (A) Schematic illustration of PSMA targeted BIND-014 docetaxel loaded polymeric nanoparticles. (B) Depiction of achievable nanomedicine attributes, red line represents the optimized properties. (C) CT scan evidencing a remarkable regression of lung metastases in 51 year male patient treated with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

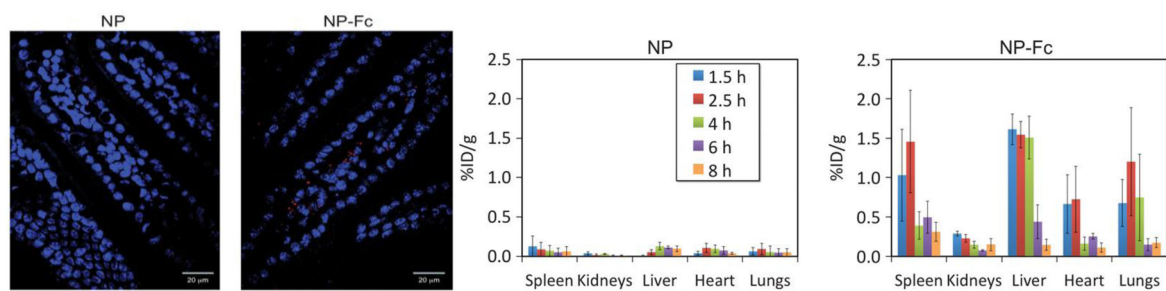


Fig. 3. FcRn targeted nanoparticles can be seen as red puncta in the confocal fluorescence images of sections of mouse duodenum (left panel). Comparison of organ localization of FcRn targeted and non-targeted PLA-PEG nanoparticles (right panel). Reproduced with permission from [136].

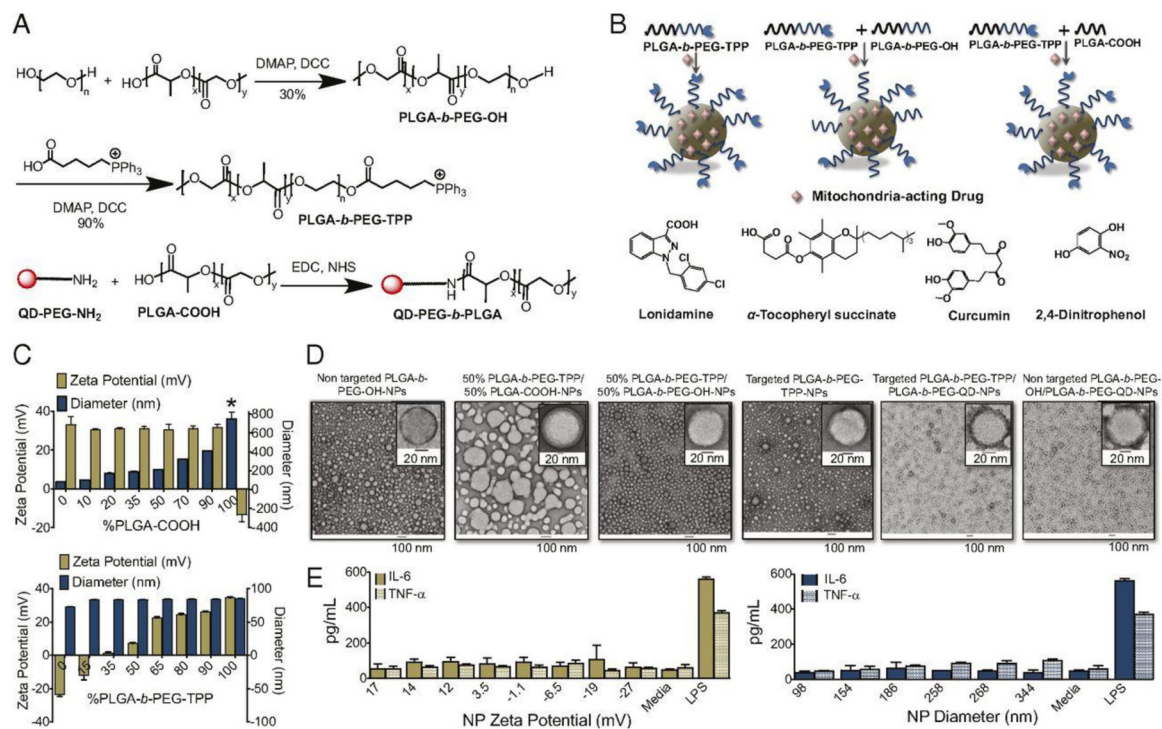


Fig. 4. (A) Synthesis of TPP end capped and QD functionalized PLGA-PEG block copolymers. (B) Schematic illustration of drug loaded targeted nanoparticles. (C) Modulation of size and zeta potential. (D) TEM images of the fabricated nanoparticles. (E) IL-6 and TNF- α secretion profiles of nanoparticles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

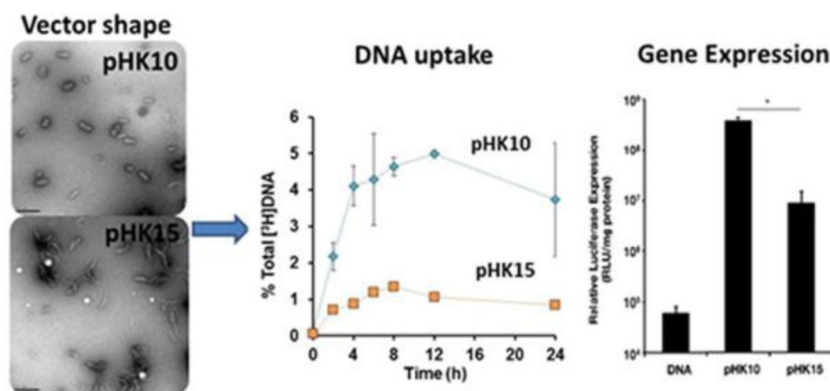


Fig. 5. TEM images revealing the effect of the number of L-lysine units on the vector shape (left). A comparison of DNA uptake capacity of pHK10 and pHK15 (middle) and gene expression (right). Reprinted with permission from [198]. Copyright (2013) American Chemical Society.

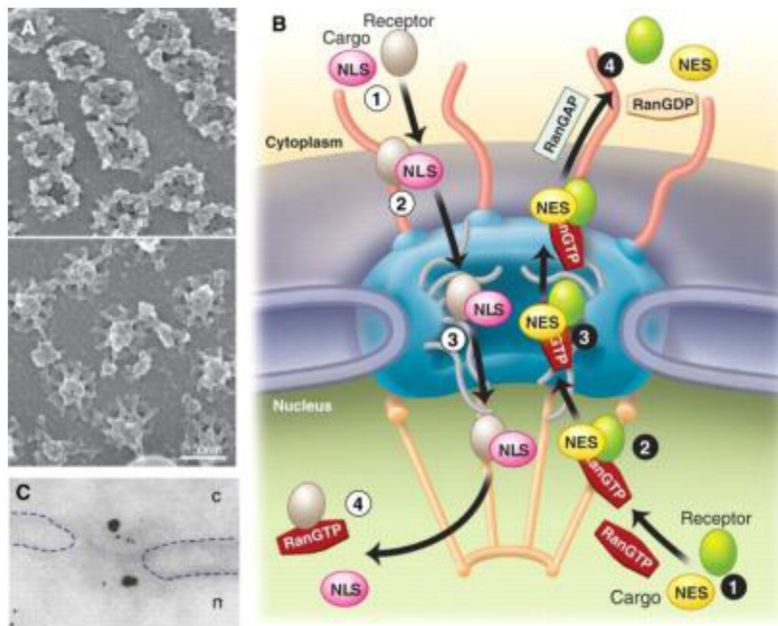


Fig. 6. (A) Scanning electron microscopic images of cytoplasmic (upper) and nucleoplasmic sides of nuclear envelope revealing the NPCs. (B) Schematic depiction of receptor mediated transportation across the NPCs. (C) Immunoelectron microscopic image of gold nanoparticles translocation between nucleus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