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Macropinocytosis as a cell entry route for peptide-functionalized and bystander nanoparticles

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Abstract

Endocytic pathways provide the primary route for therapeutic and diagnostic nanoparticles (NPs) to enter cells and subcellular compartments. A better understanding of these cell entry processes will not only aid in nanomaterial applications but also broaden our knowledge of cell biology. Among the endocytic routes, macropinocytosis has unique characteristics for engulfing NPs and other large cargo, yet its molecular machinery and involvement in NP uptake are far less characterized relative to other pathways. In this review, we summarize the current knowledge on the macropinocytic machinery, and its involvement in NP internalization. Particularly, we differentiate ligand (specifically peptide)-functionalized and unfunctionalized NPs (bystander NPs). While most of previous research focused on ligand-functionalized NPs, we showcase here a synergistic effect between these two NP types during their cell entry through receptor-mediated macropinocytosis. The regulation of NP uptake efficiency by extracellular amino acids is also highlighted in the context of interconnections between macropinocytosis and metabolic signaling. These discussions may fuel future research interests in improving NP internalization through this pathway, and open a new avenue to study the interplay among endocytosis, metabolism and nanomedicine.

Introduction

Nanoparticles (NPs) are typically defined as entities with a diameter between 1 and 100 nm. Due to their tunable physicochemical characteristics (e.g. size, shape and surface chemistry), NPs have proven useful as therapeutic and imaging agents [1]. Since the liposomal doxorubicin, Doxil, was first approved in 1995, nanomedicine has attracted great interest in both academia and pharmaceutical industry [2]. One common prerequisite for NP-formulated drugs to achieve desirable clinical outcomes is efficient entry into cells or even certain subcellular compartments [3]. Therefore, it is important to understand cellular pathways for NP entry into cells, and explore ways to increase their efficiency.

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The plasma membrane of mammalian cells is made of a lipid bilayer that separates the intracellular region from the outside environment, and regulates the movement of substances in and out of cells. It allows small molecules, such as ions and amino acids, to pass through it freely or via specific transmembrane transporters and ion channels [4]. Macromolecules, such as proteins and nanosized particles, however, generally cannot permeate the cell membrane by diffusion. Moreover, hydrophilic polymers are often coated on NPs to increase solubility in the circulation, but they form a barrier for NPs to pass through lipophilic structures (e.g. the plasma membrane). Therefore, NP internalization is mainly mediated by an active cellular process, endocytosis [5]. One type of endocytosis is phagocytosis (cell eating). This occurs in phagocytic cells, such as dendritic cells, neutrophils and macrophages, and is specialized in engulfing large solid particles (~ μm in diameter) [6]. The other type is pinocytosis (cell drinking). Generally, relatively small particles are internalized by this process, which occurs in almost all mammalian cells. Pinocytosis is further subdivided into macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis [6].

In this review, we will first provide a brief overview of endocytic pathways. Then we will focus on macropinocytosis, which has unique characteristics to mediate NP internalization. While the majority of previous studies were centered around ligand-functionalized NPs [7], we will also discuss bystander NPs, which are not equipped with cell-penetrating ligands and thus cannot enter the cells by themselves or very inefficiently. However, an important observation is that a synergistic transport exists between peptide-functionalized and bystander NPs [8]. Finally, we will review the regulation of amino acid concentration on NP internalization, and its connection to metabolic signaling and macropinocytosis machinery.

Endocytic pathways and their roles in nanoparticle uptake

The main physiological function of phagocytosis is to eliminate exogenous pathogens as well as endogenous senescent cells [9]. When NPs are systemically administered, immunoglobins, complement components and other serum proteins absorb onto the NP surface and form a protein shell, which facilitates adherence to phagocytic cells (opsonization) [10]. These opsonized NPs then bind to cell surface receptors, including Fc receptors, complement receptors, mannose receptors and scavenger receptors. The receptor-ligand interaction triggers a signaling cascade mediated by Rho-family GTPases, which involves activation of Cdc42, Rac and downstream kinases [11]. As a result, actin rearrangements induce cup-like membrane protrusion around the particle in preparation for engulfment [11]. With complete encirclement, the resulting phagosome carries the cargo throughout the cytoplasm to the lysosome. The lysosome fuses with the phagosome to form a phagolysosome for the purpose of degrading the NPs. With destruction, the NP payload is released. Relatively large particles are usually engulfed by phagocytosis, as phagosomes are sufficiently large (> 250 nm) [12]. Champion et al. described the striking effect of the particle shape on phagocytosis by fabricating six different shapes of polystyrene NPs in the range of 1 – 10 μm . They found that NPs with a high aspect ratio underwent minimal phagocytosis [13]. Due to the presence of phagocytic cells in the reticulum endothelium system (RES) organs, such as liver and spleen, considerable effort has been devoted to avoiding phagocytosis and thus increasing the accumulation of NPs at the diseased sites

[14]. Notable examples include coating NP surface with poly (ethylene glycol) (PEG) or “marker of self” CD47 peptide [15, 16].

Clathrin-mediated endocytosis (CME) is the major pathway for receptor-mediated endocytosis, which has been estimated to contribute to over 95% of endocytic flux [17]. The major structural protein of its endocytic vesicles is clathrin, which has led to the vesicle name as clathrin-coated pits. These vesicles are 60 – 120 nm in diameter [18]. The sequential steps of the CME process has been reviewed elsewhere in greater detail [18]. The endocytosed vesicles are merged into early endosomes, which are further sorted into either recycling endosomes and returned to the plasma membrane or degradative lysosomes resulting in drug and/or content release [5]. CME appears to be the most prominent mechanism for cells to engulf a wide range of NPs functionalized with ligands, such as transferrin, low-density lipoprotein and epidermal growth factor [19]. CME also provides a pathway, albeit minor, for unfunctionalized NPs provided that they possess the required physicochemical properties. Vasir et al. reported that CME-mediated uptake of poly (D, L-lactide-co-glycolide) NPs was increased with poly-L-lysine functionalization compared to those unmodified [20]. Furthermore, pH-sensitive and enzyme-cleavable modifications of NPs can be exploited as two strategies to enhance endosomal escape [5]. For example, Benyettou et al. found that by using the acid-mediated release of payloads, the NP delivery system showed significantly improved anticancer outcomes [21].

Caveolin-mediated endocytosis (CavME) is driven by flask-shaped membrane invaginations called caveolae [22]. These endocytic vesicles are 60 – 80 nm in diameter and rich in cholesterol and sphingolipids [6]. Upon specific receptor-ligand interactions, cargo, such as NPs, is concentrated in caveolae invaginations [23]. Subsequent activation of signaling molecules, including G proteins, kinases and endothelial nitric-oxide synthase, trigger caveolae endocytosis. Similar to CME, GTPase dynamin oligomerizes around the “flask” neck to pinch off membrane vesicles. The resulting cytosolic caveosomes are ultimately delivered to the Golgi apparatus and endothelium reticulum, and thereby the degradative lysosomes are avoided. Therefore, NPs can take use of this internalization mechanism to target specific organelles and avoid the hydrolytic sensitivity. For example, Xin et al. reported rod-shaped drug particles enabled efficient delivery of miRNA, which bypassed the lysosomal route in cytosol [24]. The role of caveolae is also associated with transcytosis in endothelial cells, allowing NPs to overcome the endothelial barrier in the body and increase the exposure to diseased tissues. Work by Liu et al. demonstrated that polyamidoamine dendrimer NPs modified with a rabies virus glycoprotein had a higher ability to cross the blood-brain barrier through CavME [25]. It has been reported that anionic particles and specific surface ligands, such as folic acid, albumin and cholesterol, undergo uptake by this mechanism [26, 27].

Molecular machinery of macropinocytosis

Macropinocytosis is characterized by actin-dependent formation of large-sized (200 nm - 5 μm in diameter) membrane protrusions [28]. It occurs in almost all cell types and has been implicated a diverse array of physiological functions [29]. It is commonly regarded as a nonselective pathway for cells to engulf extracellular fluid and the solutes contained therein.

Its non-selective nature is suggested by the fact that the cargo does not rely on binding to specific cellular receptors to initiate the internalization process. Macropinocytosis is distinct from other endocytic pathways mainly in the following aspects. First, there is no known structural protein to define macropinosomes (like clathrin for CME or caveolin for CavME). Second, the size of macropinosomes is heterogeneous and much larger than clathrin-coated pits and caveolae. Although the sizes of endocytic vacuoles of phagocytosis and macropinocytosis overlap to some extent, phagocytosis almost solely occurs in phagocytic cells (e.g. macrophages and dendritic cells) initiated by interactions between cellular receptors and pathogen components [9]. In contrast, macropinocytosis can be receptor-independent, accounts for fluid uptake instead of large solid particles, and occurs in almost all cell types.

Innate immune cells, such as macrophages and dendritic cells, undergo a high rate of macropinocytosis constitutively to engulf sample antigens from the nearby environment [30]. Many viruses and pathogenic bacteria were reported to promote membrane ruffling and macropinosome formation via this pathway, including adenovirus, Ebola virus and *Legionella pneumophila* [31, 32]. The activity of this process depends on extracellular calcium [33]. Depletion of calcium in the medium leads to the loss of membrane ruffles. A G protein-coupled calcium sensing receptor (CaSR) on the cell surface is responsible for calcium sensation and activation of phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC), which are involved in actin filaments nucleation [34]. Malignant cells can also adapt macropinocytosis to acquire extracellular nutrients (e.g. proteins) for survival and proliferation, which serves as a hallmark of cancer metabolism [35]. The macropinocytotic internalized proteins undergo degradation into intracellular amino acids that can fuel metabolism and cell growth [35]. Growth factors, including epidermal growth factor, platelet-derived growth factor and colony-stimulating factor-1 or tumor-promoting factor, can stimulate the activity of macropinocytosis [10]. Depletion of nutrients can drive activation of growth factor receptors in a variety of cancer cells, notable examples of which are those bearing Ras oncogenic mutations [29, 36, 37]. As the central regulator, mechanistic target-of-rapamycin complex-1 (mTORC1) coordinates these external signaling and controls the subsequent metabolic responses [28]. To be specific, growth factors activate the PI3K-Akt pathway, which then activates Rheb. Moreover, amino acids can activate two GTPases Rag and Rheb on the lysosomal surface. After being recruited by Rag, mTORC1 is directly activated by Rheb, which functions to increase protein synthesis and inhibit autophagy (Figure 1) [28].

The irregular movements of membrane ruffles initially transform into cup-like structures and then close fully to form mature macropinosomes [38]. During the initial macropinocytic cup formation, PI3K transiently converts phosphatidylinositol 4,5-bisphosphate [PI (4,5) P₂] on the cytosolic side of the membrane into phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which can be further hydrolyzed into diacylglycerol (DAG) by PLC [39]. The resulting PIP₃ spike is associated with recruitment of several guanine nucleotide exchange factors (GEFs), which can activate Rho-family GTPases, including Rac1/2 and Cdc42. These in turn promote the Arp2/3-dependent branched actin polymerization. During the cup closure into the macropinosome, Ras is considered to be crucial. It can not only regulate PI3K via its Ras-binding domain but also directly activate the formin ForG, a cytoskeletal protein

involved in actin assembly [40]. An early study showed that HeLa cells expressing a mutated Ras gene exhibited elevated levels of membrane ruffles and macropinocytosis [41]. Vacuolar ATPase (V-ATPase) trafficking to the plasma membrane is a prerequisite for ruffles in oncogenic Ras-transformed cells [42]. However, MEF cells with triple knockout of Ras still retained the capability of performing macropinocytosis [43]. It remains largely undefined whether the role of Ras can be performed by other proteins, or PIP₃ alone is sufficient to form complete macropinosomes.

After macropinosomes are fully formed, PIP₃ is sequentially dephosphorylated, while DAG is converted to phosphatidic acid (PtdOH), which continues to bolster the activation of actin nucleation [40]. Macropinosomes then move along the microtubule network and fuse with lysosomes, although recycling to the cell surface does rarely occur [28]. During cup closure, the Rab GTPase family begins to accumulate on macropinosomes and continues throughout their maturation [39]. Specifically, localization of Rab5 marks newly internalized vesicles, whereas Rab7 is associated with later phases [44]. In the early stage, macropinosomes show the ability of tubulation and shrinkage to concentrate the vesicle cargo that would require the removal of membrane and water [45]. After fusion with lysosomes in the late stage, V-ATPase accumulates on the membrane to acidify quickly the macropinosomal lumen, and lysosomal hydrolases are activated to degrade their substrates [34]. Amino acids either delivered directly into the lysosomes or generated from protein degradation can activate Rheb, which activates mTORC1 subsequently [46]. For macrophages and dendritic cells, the intracellular destinations are different from those reported in other cell types [39]. Macropinosomes in these cells often fuse with both early and late endosomes from other endocytic pathways, such as CME and CavME [32]. This may facilitate the digested peptides to bind to the Major Histocompatibility Complex II-loaded vesicles for antigen presentation.

Directing NPs to macropinocytosis as the pathway for internalization would provide several advantages over other endocytic pathways. First, its relatively large and heterogenous endocytic vesicles are more suitable to accommodate a greater capacity of NPs of various sizes and shapes than that of CME or CavME pathway [32]. Second, compared to phagocytosis, macropinocytosis provides a universal route into a much broader range of cell types. Third, a high rate of macropinocytosis occurs constitutively in antigen-presenting immune cells and many cancer cells, which can be advantageous for certain NP applications. One such example is NP-based vaccination [86]. The strong macropinocytotic activity in immature dendritic cells allows efficient engulfment of peptide antigens conjugated to NPs, which boosts vaccination efficacy. Macropinocytic uptake of nab-paclitaxel, an albumin-formulated NP carrying paclitaxel, was also shown to induce the polarization of tumor-associated macrophages towards an immunostimulatory phenotype [47]. This result is significant as it indicates that immunity can be modulated through this pathway. As the functional relevance of macropinocytosis is linked with nutrient acquisition, its metabolic role is highlighted under pathological conditions. NP associated with proteins, such as albumin-bound fatty acids, are more likely to enter cancer cells via macropinocytosis [87]. A lipoprotein-based NP system carrying miRNA in the core achieved efficient accumulation and therapeutic effect in gliomas through CXCR4 receptor-stimulated macropinocytosis [48]. Although the large size of macropinosomes is considered to favor uptake of nano- and

micro-sized particles, many NPs have been reported to utilize more than one endocytic route [6]. For instance, a cationic and hydrophobic glycol chitosan NP with the mean diameter of 359 nm could be internalized by multiple distinct pathways, including CME, CavME and macropinocytosis [49]. To trace macropinocytosis, 70kDa Dextran (Dextran thereafter if not indicated otherwise) is a commonly-used fluid-phase marker as its large hydrodynamic size excludes the possibility of being engulfed by CME and CavME [50].

Peptide-functionalized nanoparticles and macropinocytosis

In order to enter the cells, NPs are often functionalized with cell-penetrating ligands. Notable examples are cell-penetrating peptides (CPPs), a family of short peptides (typically 5 – 30 amino acids) capable in crossing cellular membranes [51]. Coupling cargo with CPPs by either covalent bonds or noncovalently electrostatic/hydrophobic interactions enables the conjugates to access their intracellular targets, thereby increasing the therapeutic effect [52]. Based on the physical-chemical properties, CPPs can be divided into three classes: cationic, amphipathic and hydrophobic peptides. Cationic CPPs, mainly rich in arginine and lysine, were discovered first and consist of the majority of known CPPs [51]. They have been widely used in carrying diverse cargo types into cell, such as proteins, nucleic acids, small molecule drugs and NPs [53].

Not surprisingly, the cell entry mechanisms of cationic CPPs are also relatively well-characterized [54, 55]. One such example is CendR peptides. These peptides contain a positively charged R/KXXR/K motif at their C-terminus (thus *C-end Rule*, CendR) [56]. They were discovered using *in vivo* phage display, a powerful technology to identify tissue-homing peptides under physiological conditions [57]. The prototypic tumor-homing CendR peptide, iRGD, exhibits two unique properties. First, iRGD can selectively recognize tumor vasculatures and penetrate across the vessel wall and deep into the extravascular tumor tissues [58]. This tumor-penetrating property applies to the peptide itself and its coupled cargo, which can range from small molecules to NPs. Second, iRGD can also enhance the tumor penetration and accumulation of co-administered but not covalently linked cargo [59]. This property is termed as the “bystander effect”. The implication of this discovery is highly significant, because the delivery and antitumor efficacy of clinically approved drugs can be enhanced by simple co-administration instead of chemical modifications. The tumor penetration and bystander effect were found with other CendR peptides as well [79, 80, 81, 82].

CendR peptides were demonstrated to invoke endocytosis upon binding to the cell surface receptor, neuropilin-1 (NRP1) [60]. Neuropilins are non-tyrosine kinases of 120 – 130 kDa and are widely expressed in various tissue types, including endothelium and solid tumors [61]. Their physiological functions are implicated in angiogenesis and neuron development. NRP1, and NRP2 to a lesser extent, serve as receptors or co-receptors for multiple growth factors, for example, vascular endothelial growth factor (VEGF)-A isoforms [62]. Particularly, VEGF-A165 contains an active CendR motif [63]. A prototypic CendR peptide, RPAPAR, was shown to enhance vascular permeability upon subcutaneous injection similarly as VEGF. This led to the initial but incorrect speculation that the bystander effect mechanism was achieved by increasing the vascular permeability locally in tumors.

However, later studies demonstrated CendR peptides use an active transport process for vascular and tissue penetration, rather than passive diffusion through intercellular gaps in highly permeable vessels, [56, 58, 64]. Liu et al. directly visualized iRGD-mediated vascular penetration in tumors by transmission electron microscopy (TEM) and further proved that iRGD-NP complexes crossed the endothelium via transcytosis (Figure 2A) [65].

Pang et al. performed a genome-wide RNA interfering (RNAi) screen to identify genes and signaling pathways that regulated the cell entry of CendR cargo [66]. RPARPAR was conjugated to silver NPs (R-AgNPs) as the model CendR cargo [66]. AgNPs were used to take advantage of etching technology that can be used to dissolve extracellular AgNPs rapidly, while internalized NPs remain intact [67]. The screen identified macropinocytosis genes, such as Rac1, Cdc42, Rab5, some members of PI3Ks and PLCs, but not clathrins or caveolins [66]. The uptake of R-AgNPs was sensitive to macropinocytosis inhibitors and also depended on CendR-NRP1 ligation. Ultrastructurally, the endocytic vesicles resembled macropinosomes, which were over 250 nm in diameter and showed irregular shapes (Figure 2B). When co-administered with Dextran, R-AgNPs exhibited limited colocalization, highlighting a difference between CendR-NRP1 initiated and conventional receptor-independent macropinocytosis. Together, these results provide strong evidence that CendR-mediated cell entry occurred through receptor (NRP1)-mediated macropinocytosis.

Another finding was that CendR-mediated endocytosis was governed by nutrient sensing and translation regulatory pathways, especially mTOR signaling [66]. Genome screen hits included members of mTOR complex 1 and 2, up- and down-stream targets, such as PI3K/Akt-related genes and translational initiating factors (EIF3s and EIF4s). Specifically, mTOR inhibition or deprivation of extracellular nutrients (e.g. glucose and amino acids) enhanced the cell entry efficiency of CendR cargo, including NPs and proteins (Figure 2C) [66]. The response of CendR endocytosis to mTOR inhibition and nutrient deprivation was different from that of CME, CavME as well as conventional macropinocytosis traced by Dextran (Figure 2C). This further supports the possibility of receptor (NRP1)-mediated macropinocytosis being a novel endocytic pathway [66]. Despite previous studies on the activation of mTOR pathway by amino acid influx mediated by macropinocytosis, this was among the first reports showing the regulation of macropinocytosis by mTOR or nutrient availability. The upregulated expression of NRP1 on the cell surface partly accounts for this phenomenon, but other possible mechanisms remain to be investigated [66].

In terms of physiological relevance, NRP1-mediated bulk transport pathway may be activated for essential material acquisition in solid tumors, where oxygen and nutrients are often limited [68]. CendR peptides can therefore commandeer this pathway for delivery therapeutic cargo. The nutrient regulation is also consistent with previous reports on the connection between macropinocytosis and mTOR signaling as discussed above. The underlying mechanism and physiological relevance are potentially of significance to both cell biology and nanomedicine research.

Besides CendR peptides, other cationic CPPs also use receptor-mediated macropinocytosis to transport NPs into cells. Transactivator of transcription (TAT), derived from human immunodeficiency virus-1 (HIV-1), was first found in 1988 and later considered as the

prototype cationic CPP [69]. Pang et al. coated NPs with TAT made of L- and D-amino acids and found that TAT-NPs were taken up in the macropinosome-like structures (> 250 nm) (Figure 2D) [70]. This engulfment relied on TAT interaction with its receptors, heparan sulfate proteoglycans (HSPGs). Consistently, TAT-fusion proteins were shown to transduce into cells via macropinocytosis and exhibit endosomal escape [71]. This HSPG-mediated macropinocytosis was also reported to mediate internalization of albumin conjugated with a TAT-like peptide, polyarginines [72]. Multiple endocytic pathways and even direct translocation across the plasma membrane have been proposed as the internalization mechanism for TAT peptide [73, 74, 75]. In contrast, when TAT peptides are multiplexed on proteins or NPs, macropinocytosis was the main mechanism [70, 76]. The difference between monomer peptide and its multiplexed form on NP may contribute to distinct receptor interactions and eventually determines the endocytic route.

NRPs and HSPGs are present in immune cells and may involve in phagocytosis as well [88]. However, the processes observed above appear distinct from receptor-mediated phagocytosis. Phagocytosis normally involves membrane wrapping of a single solid particle [85]. The cell membrane is in close proximity to the particle surface. However, the size of peptide-functionalized NPs in these studies was much smaller than the diameter of endocytic vesicles, and multiple NPs were simultaneously engulfed within a single endocytic vacuole (Figure 2B and 2D) [66, 70]. Additionally, tumor cell lines were primarily used to perform these studies. As such, this process is more related to receptor-mediated macropinocytosis rather than phagocytosis. On the other hand, the main features setting this process apart from traditional macropinocytosis, include receptor dependence and distinctive responses to mTOR signaling and nutrient availability, as discussed above. One key factor contributing to these differences may be the internalization dynamics. In these studies, the authors used a relatively short time (~1h) for internalization, while a much longer time (~24h) was shown to be required for the efficient uptake of unfunctionalized NPs [89]. Considering the blood flow rate and the time frame for circulatory cargo to encounter a certain tissue (e.g. tumor), it is unlikely for systemically administered NPs to have such a long time together with target cells for efficient internalization. Therefore, this receptor-initiated macropinocytosis will be of greater use to improve the efficacy of NPs *in vivo*.

Other than cell entry, an *in vitro* cellular assay was established to study the possibility of transcellular transport of CPP-NPs [66]. This study proved that both CendR- and TAT-functionalized NPs could be exported from one cell and be re-absorbed into another. This cell-to-cell transfer was demonstrated to be mediated by membrane-enclosed structures, as etching did not significantly lower the transcellular efficiency [66]. Here, this result provides a possible mechanism for CendR-mediated tumor penetration and raises an intriguing question regarding the final destination of engulfed cargo by this pathway. Future investigations on this process are needed to shed light on cellular transport machineries that are responsible for not only internalizing cargo into cells but also transporting across the tissues of multilayered cells.

Nanoparticle uptake in the bystander manner via receptor-mediated macropinocytosis

Receptor-mediated macropinocytosis also provides a possible mechanism for the bystander effect [56]. The bystander effect is the phenomenon when the cell entry of one molecule (an active ligand) enables the internalization of co-administered cargo (bystander cargo). This is most convincingly demonstrated when the bystander cargo has little or no ability to enter the cells themselves and has no physical or chemical interaction with the active ligand. Sugahara et al. first tested the bystander effect of iRGD by co-injection with a variety of anticancer drugs [59]. The tumor accumulation and antitumor efficacy were significantly enhanced for a small molecule drug (doxorubicin), nanoparticles (nab-paclitaxel and liposomal doxorubicin), and an antibody (trastuzumab) when used together with iRGD. It should be noted that this effect circumvents the limited availability of target receptors on the cell membrane, since bystander cargo are engulfed through a bulk transport system [59]. Since then, several preclinical studies have taken advantage of this effect to increase the specificity and therapeutic effects of various drugs [77, 78].

Besides iRGD, other CendR peptides have been shown to exhibit the bystander activity. LyP-1 is a tumor-homing peptide with a cryptic CendR motif and preferentially binds to tumor macrophages after extravasation [56]. Both LyP-1 and its linear truncated form tLyP-1 were reported to generate the bystander effect [79, 80, 81]. Timur et al. combined LyP-1 and doxorubicin hydrochloride in a self-microemulsifying drug delivery system that showed enhanced *in vitro* cytotoxicity and reduced tumor growth *in vivo* [79]. When tLyP-1 was co-administered with tumor-targeted NPs, deeper penetration into brain and greater therapeutic effects were observed in mice bearing glioma [80]. Another example is iNGR, a *de novo* designed tumor-penetrating peptide with the C-terminal consensus motif [82]. Co-administration of iNGR with doxorubicin enhanced the anticancer efficacy. A more thorough review of peptides with the bystander activity can be found elsewhere [56].

Considering potential therapeutic value, it is of great importance to decode the cellular mechanism for this bystander activity. Despite intensive characterization *in vivo*, the bystander effect of CendR peptides has rarely been studied *in vitro*. A closest phenomenon came from cell entry of TAT peptide. Kaplan et al. showed that co-administration with TAT peptide could increase the cellular uptake of Dextran (Figure 3A) [73]. TAT and Dextran were not linked chemically or physically so Dextran is likely a bystander payload. Fluid-phase macropinocytosis is believed to account for this improved transport, because the formation of macropinosomes may sweep nearby cargo in bulk into the large endocytic vesicles (> 200 nm in diameter) [56]. Table I provides the list of the peptides that have served as active ligands. Table II summarizes the studies that have shown an elevated intracellular delivery in the bystander manner.

Although it is challenging to stimulate the bystander effect with free CPPs, a recent breakthrough has been made with CPP-conjugated NPs. Using TAT-functionalized NPs, Wei et al. investigated the bystander activity on a variety of cargo, including NPs, albumin and Dextran [8]. The tested NP types included AgNPs, gold nanoparticles (AuNPs), iron oxide nanoparticles (IONPs) and quantum dots (QDs). The authors found that TAT-NP co-

administration significantly enhanced the cellular uptake of all four NP types in the bystander manner (Figure 3B) as this effect was not due to TAT exchange between NPs or any interactions between the two NPs. This phenomenon was observed when TAT was conjugated to different NP types, in multiple cell line, as well as *ex vivo* tissues and *in vivo* settings. Interestingly, the bystander effect induced by TAT-NPs did not apply to common fluid markers, albumin and Dextran. In comparison, TAT peptide exhibited bystander activity to fluid markers and some types of bystander NPs but not all (Figure 3B). This cargo selectivity highlighted the difference between bystander uptake processes invoked by free peptide and its multivalent form on NP. Moreover, the level of bystander activity showed dependence on the composition of TAT-NPs and cell types, and the concentration of either TAT-NPs or bystander NPs.

The authors further showed that the bystander uptake induced by TAT-NPs used receptor-mediated macropinocytosis [8]. Initiation of this process relied on the interaction between TAT-NPs and cellular receptors, HSPGs. Inhibitor treatment demonstrated that TAT-NP-induced bystander uptake was primarily sensitive to macropinocytosis inhibitors. TEM studies provided the visual evidence that bystander cargo are located in the same cup-like macropinosomes (> 200 nm in diameter) with TAT-NPs (Figure 3C). Inside the cells, bystander NPs co-localized almost exclusively with TAT-NPs. Together, the results support that TAT-NPs invoked receptor (HSPG)-mediated macropinocytosis to engulf bystander NPs.

When studying the cell entry of NPs, especially the synergy between different NP types, an important consideration is to avoid the contribution of NP aggregates. The aggregation will not only render the above conclusion invalid and invoke different internalization routes but also induce unwanted cytotoxicity. *In vivo*, NP aggregation may influence biodistribution and cause severe adverse effects, such as capillary blockage [90]. Uptake of solid NP aggregates is primarily reported in phagocytosis, which involves internalization of two-phase (water-solid) materials [91]. One study investigated the clustering state of NPs which entered the cells via multiple fluid-phase endocytic pathways, including macropinocytosis [92]. They found that NPs appeared separately in the early stages of endocytosis but only clustered during vesicular maturation (e.g. late endosome and lysosomes). In the above studies, various procedures have been taken to ensure no aggregation. Hydrophilic polymers (e.g. PEG) were coated on NPs to promote dispersion. Multiple methods were used to ensure that there was no physical interaction between CPP-NPs and bystander NPs [8]. Based on TEM images, NPs are well dispersed at both early and late stages of endocytosis, further supporting that this phenomenon does not arise from NP aggregates (Figure 2B, 2D and 3C) [8, 66, 70].

Inspired by previous studies, the authors investigated the impact of extracellular amino acids (AAs) on the bystander effect [8]. They found that among all 20 natural AAs, there was only one AA, cysteine, whose concentration affected bystander activity (Figure 3D). TAT-NP-mediated bystander uptake was much higher in the cysteine-containing medium than that without cysteine. This cysteine regulation occurred in all tested NP types (as active ligands or bystander cargo), a variety of cell lines, and under *ex vivo* and *in vivo* conditions. Cysteine variants, cystine and glutathione (GSH), had similar effects. Blockage of cysteine

transporter with an inhibitor significantly reduced bystander activity, indicating that this effect depended on cysteine uptake into cells and the intracellular redox status. Interestingly, the authors observed little or modest cysteine effect on TAT-NP uptake itself [8]. Although these results show that the uptake of functionalized and bystander NPs was not merely coincidental engulfment, further investigation is needed to dissect the interconnection between individual amino acids and endocytic machinery.

Regarding bystander uptake and amino acid regulation, there are more questions than answers. First, the physiochemical properties of NPs that determine their bystander activity are far from being understood. The particle size may be an important factor since QDs, with the smallest size, exhibited the lowest activity as bystander cargo [8]. This may also explain the above cited result of albumin and Dextran. Previous research that focused on NP-cell interactions reported that 50-nm spherical AuNPs showed the highest uptake compared to any rod-like shapes in the range of 30 – 90 nm diameter [83]. However, it remains a question whether this optimal size and shape apply to NPs as bystander cargo. Other parameters of NPs, such as rigidity and surface charge, may contribute to bystander efficiency as well. Second, the nature and molecular components for macropinosomes specifically responsible for bystander uptake remain to be investigated. This belongs to the even larger question in cell biology, as the structural marker of macropinosomes is still unknown [29]. Exploring of this aspect may reveal how bystander cargo are engulfed into the same vesicles as TAT-NPs and how this endocytic process drives cargo selectivity. Third, to our best knowledge, Wei et al is the first study to show the regulation of endocytosis and NP uptake by an individual amino acid. Cysteine and its derivative, GSH, are well known for protecting cells from oxidative stress and reactive oxygen species (ROS) [84]. Although the exact mechanism connecting cysteine with bystander uptake remains to be studied, ROS and oxidative signaling are speculated to interfere with macropinocytosis activity through interactions with PI3K-Akt and mTOR pathways. The cysteine concentration did not influence TAT-NP internalization, signifying a delicate distinction between functionalized and bystander NPs, even though they both enter the cells via receptor-mediated macropinocytosis. Decoding this disparity will aid in a better understanding of macropinocytosis, its cargo selectivity and metabolic regulation.

Concluding Remarks

The molecular machinery involved in macropinocytosis has been described with an emphasis on receptor-mediated macropinocytosis process. This pathway is used by ligand-functionalized and bystander NPs for cell entry, which has important therapeutic consequences. Bystander uptake presents a useful way to synergize internalization of two NP types and circumvent the limitation of receptor availability on the cell surface [56]. These investigations also unveil several novel properties, including cargo selectivity and amino acid regulation. Addressing the details of this transport mechanism will advance our knowledge on macropinocytosis in general, help identify molecular signatures for macropinosomes, and clarify the factors determining bystander uptake efficiency and cysteine regulation. Such efforts will further improve the cell entry and hold the promise of advancing therapeutic efficacy of nanomedicine.

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Highlights

This review summarizes the macropinocytosis machinery and its internalization of nanoparticles, with special focus on receptor-mediated macropinocytosis.

This review describes a synergistic entry of peptide-functionalized and bystander nanoparticles into cells through receptor-mediated macropinocytosis.

This review highlights the regulation of nanoparticle uptake efficiency by extracellular amino acids, which opens up a new avenue to study the interplay among drug delivery, endocytosis, nutrient availability and metabolic signaling.

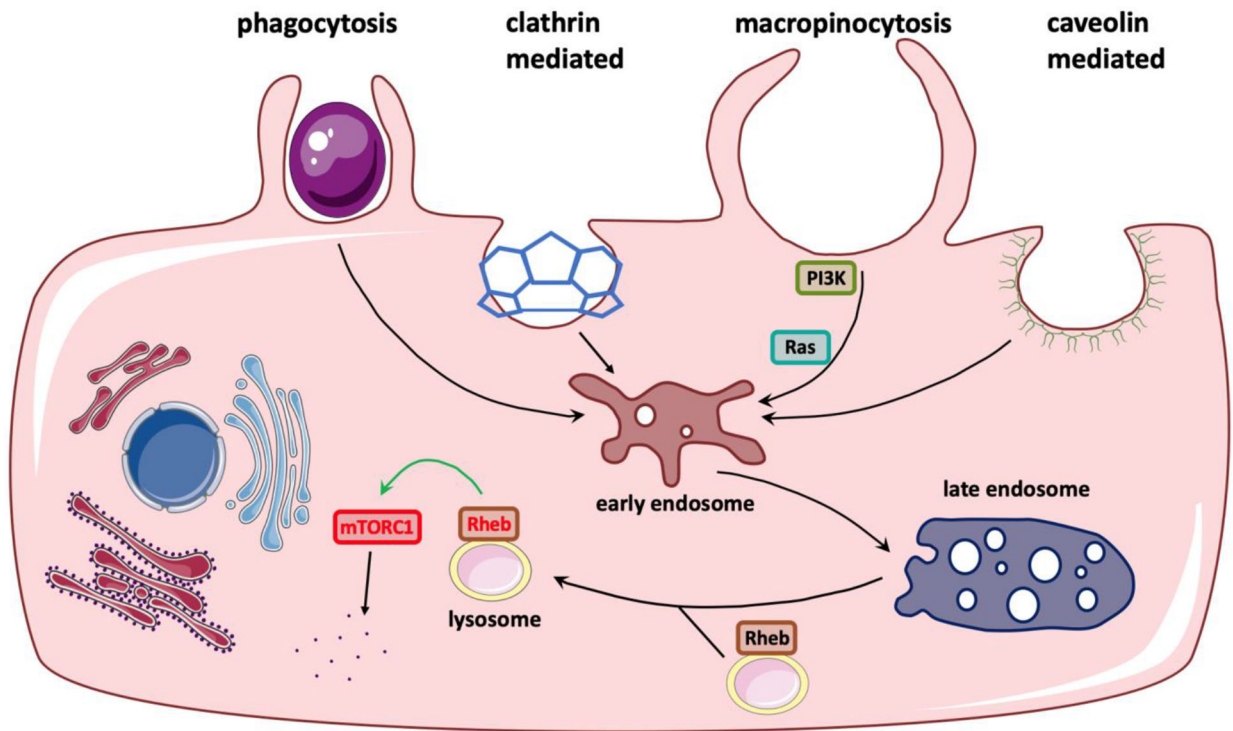


Figure 1. Endocytic pathways and macropinocytosis-mediated activation of mTOR signaling. NP enter cells via multiple mechanisms of endocytosis, including phagocytosis, clathrin-mediated, caveolin-mediated, or macropinocytosis. PI3K is recruited to enhance PI (4,5) P₂ conversion to PIP₃ which accumulate in macropinocytic cups. Ruffles and early macropinosomes are active sites for Akt signaling, resulting in direct Rheb activation on the lysosomal surface. Formation of complete macropinosomes relies on Ras. As the central regulator of cell growth, mTORC1 is then activated by Rheb.

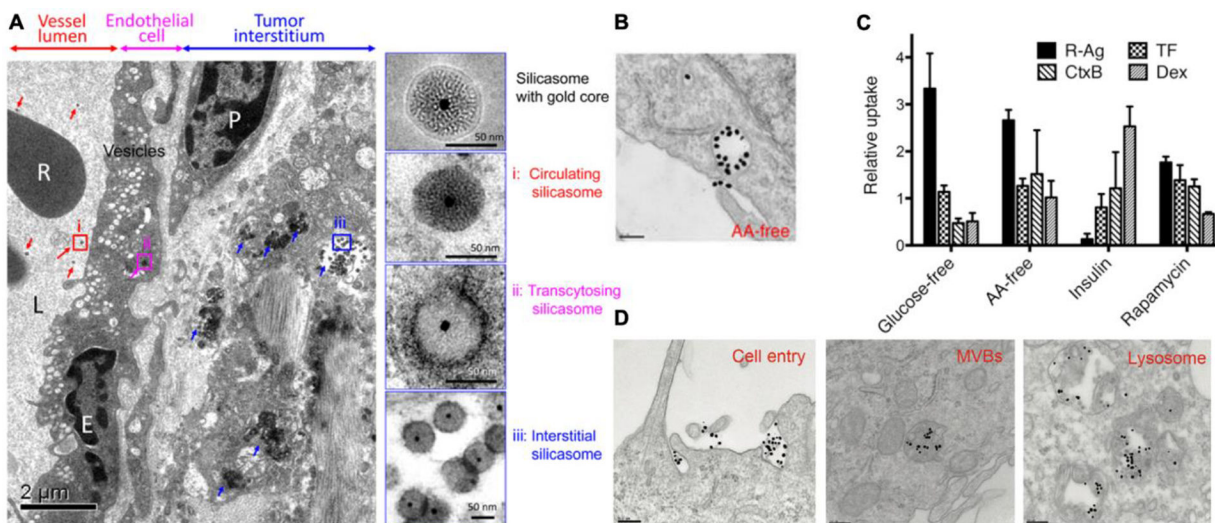


Figure 2. Receptor-mediated macropinocytosis and the uptake of CPP-functionalized nanoparticles

(A) TEM images shows that CendR peptide induced a transcytosis for NP vascular penetration. Silicasome NPs co-administered with iRGD appeared in (i) the blood vessel lumen (red arrows), (ii) transport vesicles in endothelial cells (pink arrows) and (iii) the tumor matrix (blue arrows). Right panels were magnified regions (i) through (iii). R, red blood cell; E, endothelial cell; P, pericyte. Scale bar = 2 μ m (left panel) or 50 nm (right panel). The image is adapted from [65].

(B) RPARPAR coated gold NPs (dense dark dots) were observed to be internalized into macropinocytosis-like vacuoles, which were evident in the medium depleted of amino acids. Scale bar = 200 nm. The TEM image is adapted from [66].

(C) The cellular uptake of endocytic tracers (indicated on the upper left corner of the figure) responds differently to environmental cues. The y-axis shows the cellular uptake of each tracer relative to that in the complete cell culture medium (glucose- or AA-free) or treated by control solvents (insulin or rapamycin). The image is adapted from [66].

(D) Gold NPs coated with TAT (dense dark dots) were observed to be engulfed by macropinocytosis-like vesicles, and sequentially transported through the indicated compartments (from left to right). Scale bar = 200 nm. The image is adapted from [70].

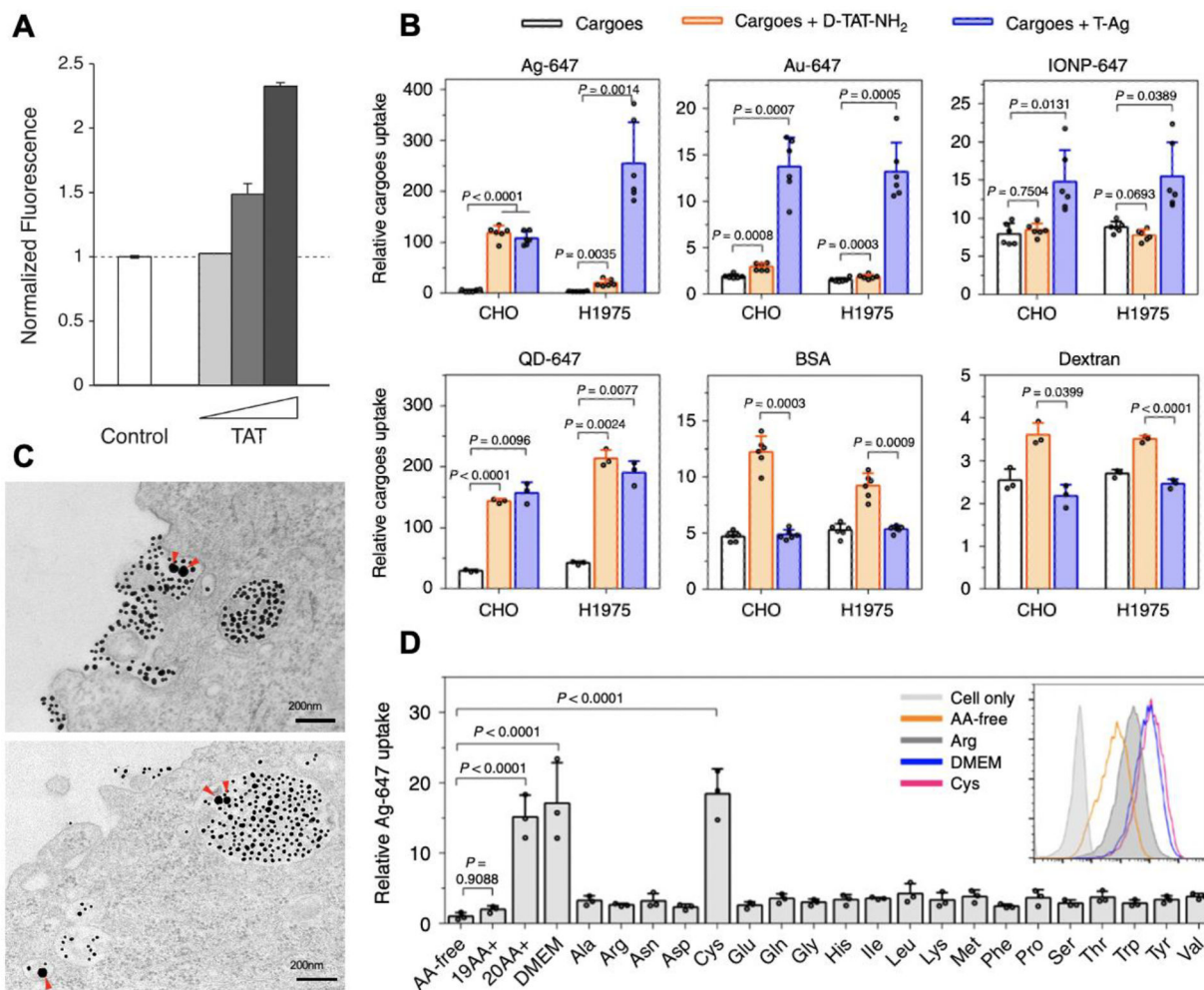


Figure 3. Receptor-mediated macropinocytosis and bystander uptake

(A) TAT peptide stimulated the bystander uptake of Dextran. The image is adapted from [73].

(B) The bystander uptake of the indicated bystander cargo by TAT peptide (orange bars) and TAT-NPs (blue bars). The image is adapted from [8].

(C) TEM images shows that TAT-coated gold NPs (smaller dark dots) and bystander gold NPs (larger dark dots, pointed by red arrows) are engulfed together by macropinocytosis-like vesicles. Scale bar = 200 nm. The image is adapted from [8].

(D) Extracellular cysteine stimulated TAT-NP-induced bystander uptake. Cells were incubated with TAT-NPs and bystander AgNPs in the culture media containing various amino acid compositions: no amino acid (AA-free), the indicated amino acid alone (e.g. Cys, Arg), 19 amino acids without cysteine (19AA+) and all the amino acids (20AA+). TAT-NP-mediated bystander uptake is much higher in the media containing all 20 AAs or only cysteine, than that in the media without Cysteine. The image is adapted from [8].

Table I.

Examples of “bystander effect” peptides, their sequences and origins

Ligand	Sequence	Origin	Reference
TAT	YGRKKRRQRRR	HIV protein	[8, 73]
Polyarginines	Rn	Chemical synthesis	[72]
iRGD	CRGDKGPDC	Phage display	[59, 77, 78]
LyP-1	CGNKRTRGC	Phage display	[79]
tLyP-1	CGNKRTR	Phage display	[80, 81]
iNGR	CRNGRGPDC	Chemical synthesis	[82]

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Table II.

Examples of bystander uptake under preclinical development

Bystander Inducer	Description	Result	Reference
TAT	TAT co-incubation could provoke the uptake of the macropinosytosis marker 70kDa Dextran.	<i>in vitro</i>	[73]
TAT-NPs	NPs conjugated with TAT could induce bystander uptake of all NP-type cargo but not common fluid markers, the activity of which was regulated by extracellular cysteine presence both <i>in vitro</i> and <i>in vivo</i> .	<i>in vitro & in vivo</i>	[8]
Polyarginines	TAT-fused albumin induced macropinocytosis through interactions with HSPG and CXCR4 to activate PKC/PI3K/JNK/mTOR signaling pathways.	<i>in vitro</i>	[72]
iRGD	iRGD could facilitate the delivery of small molecule, nanoparticle and antibody into tumors when administered as a combination therapy.	<i>in vivo</i>	[59]
	Despite little enhancement of cytotoxicity <i>in vitro</i> , co-administered iRGD promoted the delivery of PLGA nanoparticles selectively into tumors <i>in vivo</i> .	<i>in vitro & in vivo</i>	[78]
LyP-1	The LyP-1-containing self-microemulsifying formulation not only increased <i>in vitro</i> cytotoxic effect of co-administered doxorubicin hydrochloride, but also achieved preferential accumulation in tumors via intraperitoneal injection.	<i>in vitro & in vivo</i>	[79]
tLyP-1	Following tLyP-1 co-administration, tumor-targeted NPs displayed improved cytotoxicity of the loaded paclitaxel and deeper penetration into the glioma parenchyma.	<i>in vitro & in vivo</i>	[80]
iNGR	iNGR enhanced accumulation and efficacy of co-administered doxorubicin by triggering the tumor-specific penetration.	<i>in vivo</i>	[82]