Expert Opinion

Gateways for the intracellular access of nanocarriers: a review of receptor-mediated endocytosis mechanisms and of strategies in receptor targeting

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Importance of the field: The last 10 years have seen a dramatic growth in understanding and controlling how complex, drug-loaded (nano)structures, as well as pathogens, or biopharmaceuticals can gather access to the cytoplasm, which is a key step to increasing the effectiveness of their action.
Areas covered in this review: The review offers an updated overview of the current knowledge of endocytic processes; furthermore, the cell surface receptors most commonly used in drug delivery are here discussed on the basis of their reported internalization mechanisms, with examples of their use as nanocarrier targets taken from the most recent scientific literature.
What the reader will gain: Knowledge of molecular biology details is increasingly necessary for a rational design of drug delivery systems. Here, the aim is to provide the reader with an attempt to link a mechanistic knowledge of endocytic mechanisms with the identification of appropriate targets (internalization receptors) for nanocarriers.
Take home message: Much advance is still needed to create a complete and coherent biological picture of endocytosis, but current knowledge already allows individuation of a good number of targetable groups for a predetermined intracellular fate of nanocarriers.

Keywords: clathrin, endocytosis, nanocarriers, nanoparticles, targeted drug delivery

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1. Introduction

A 'nanocarrier' is virtually any colloidal object (dimension ranging from nanometers to a few micrometers) capable of hosting a pharmacologically active principle; this definition encompasses not only organic and inorganic nanoparticles, vesicles/liposomes, micelles, but also soluble macromolecular carriers of appropriate dimension.

The interfacial phenomena involving both the nanocarrier and the cellular surface determine the possibility and the modality of the cell uptake and any further form of intracellular transport, thereby significantly affecting the efficacy of encapsulated drugs [1]. More specifically, uptake may be caused by simple physical proximity to the membrane, or by more or less specific interactions with receptors on the cell surface, giving rise to different internalization paths [2]. As a feature common to all cases, the cell membrane will invaginate in order to engulf these colloidal objects together with a variable amount of extracellular fluid and enclose them into an intracellular vesicle, which will take the generic name of early endosome. The formation of early endosomes is a process generically referred to as endocytosis,

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Article highlights.

- The paper reviews the different kinds of uptake mechanism, specifically focusing on clathrin-dependent and clathrin-independent receptor-mediated endocytic processes.
- In targeted drug delivery, the most commonly used receptors are transferrin, receptor tyrosine kinases and lectins (mostly or exclusively clathrin-dependent uptake), cell adhesion molecules and cell-penetrating peptides (mixed dependence, including clathrin-dependent and clathrin-independent mechanisms), and folate receptor (predominantly caveolin-dependent uptake).
- Several endocytosis inhibitors are available to more or less selectively knock-off individual mechanisms and individuate the mode of uptake for a specific nanocarrier. However, they also bear non-negligible side effects. siRNA-mediated inhibition may lead to better results.

This box summarizes key points contained in the article.

but several structurally different endocytic processes can be recognized and are reviewed in the next section. As a common point, the intracellular structure formed is a sort of 'sorting station' for all substances internalized, leading to their degradation, translocation into other cytoplasmic compartments, or recycling towards the extracellular space [3]; its fate, therefore, is an important determinant of the successful delivery of a therapeutic payload. For example, endosomes often mature into increasingly acidic vesicles, which may or may not fuse with lysosomes, where hydrolytic and enzymatic reactions may lead to the complete destruction of the endocyted material [4,5]. However, responsive agents may interfere with this mechanism at different stages, leading, for example, to a less aggressive compartment that allows microbial survival, as in the case of *Mycobacterium tuberculosis* [6] or *Helicobacter* pylori [7], or in alternative exploit acidification to cause cytoplasmic release by means of a pH-dependent endosomal disruption, as happens for most non-viral carriers of nucleic acids [8].

The most common mechanisms of endocytic uptake are reviewed here and then they are associated with some of the most common receptors exploited in targeted drug delivery. The next challenge is to embed this information in the design of nanocarriers: this will allow the intracellular fate of a drug to be directed by driving a carrier to bind specific receptors not only with the aim of targeting certain cells, but also to follow a clear intracellular pathway.

Overwhelmingly, the results of *in vitro* experiments are discussed. However, these data provide a merely qualitative picture in view of an *in vivo* administration: nonspecific uptake of the nanocarriers by phagocytic cells during their circulation, or specificities in their biodistribution, may overwhelm the efficacy of a specific ligand. A decreased nonspecific uptake and a prolonged circulation of nanocarriers can be achieved, providing their surface with 'stealth' layers, for example, made of poly(ethylene glycol) (PEG) chains; however, their presence adds another variable to the system, as these layers may conceal, sterically hinder or at least modulate the activity of receptor-targeting ligands.

2. Gateways to cellular access

All endocytic processes are characterized by a few common features, which are preserved across eukaryotic evolution [9], for example the formation of intracellular membranes in vesicular form from the plasma membrane through its transient budding, and some form of involvement of cytoskeletal elements, such as actin [10], which is connected to the lipidic membranes [11] through the presence of some regulatory proteins, such as dynamin (a GTPase, which is capable of hydrolyzing guanidine triphosphate and using the resulting energy for the contraction of its coil) [12] or cortactin (an actin-binding protein capable of stimulating its polymerization) [13]. In addition, most endocytic processes also show some similarities to vesiculation mechanisms taking place in the *trans*-Golgi network [14].

Major differences, however, can be recognized, and allow the broad categorization of endocytic phenomena into two qualitatively different classes, depending on the physical state of the internalized objects: the first one is termed phagocytosis, which corresponds to the uptake of large, solid or solid-like bodies, for example, bacteria, debris from an implant, collagen fibers, and so on; the second one is pinocytosis, which is a general name for the uptake of a volume of fluid, possibly containing dispersed material. Pinocytic processes are then subdivided further into mechanisms that allow the uptake of large volumes of extracellular fluids (macropinocytosis and circular dorsal ruffles) and those that govern the uptake of small volumes; the latter ones are then finally divided into clathrin-dependent and non-clathrin-dependent mechanisms [15]. Hereafter, the essential features of most of them are reviewed.

2.1 Phagocytosis

Phagocytosis in mammals is essentially limited to a restricted numbers of cells, that is, macrophages, monocytes, dendritic cells and neutrophils, although eosinophiles [16], osteoclasts [17] and oligodendrocytes/microglia [18] have phagocytic abilities too.

On the other hand, pinocytic uptake is virtually ubiquitous, although its details are largely dependent on the cell kind. As a result of its structural variety, pinocytosis is subdivided further into several subclasses, that is, macropinocytosis, circular dorsal ruffles, clathrin-mediated endocytosis, caveolae-mediated endocytosis and several clathrin- and caveolae-independent endocytic mechanisms [19], which are depicted in **Figure 1** and reviewed hereafter.

2.2 Macropinocytosis

Macropinocytosis, in a first approximation, can be described as an often non-selective mechanism based on the formation of membrane protrusions that surround and take up a portion

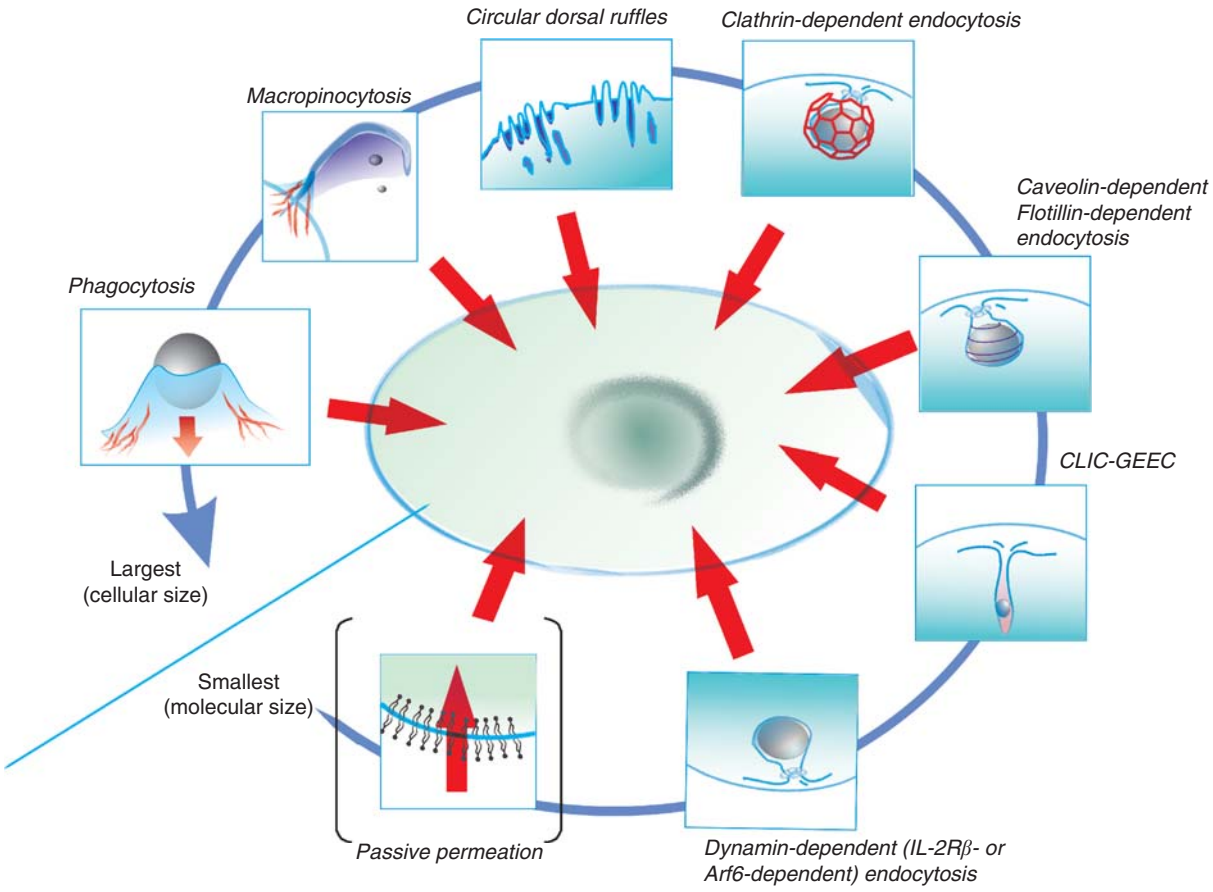

Figure 1. Different portals of entry into the cell. In the figure, the internalized volume (solid or liquid mass) progressively increases in an anticlockwise fashion, starting from a molecule-based uptake mechanism, such as passive permeation, and ending with the phagocytosis of large objects, such as whole cells. It is worth noting that passive permeation through phospholipid bilayers is substantially excused for macromolecules or nanocarriers, essentially owing to entropic reasons (negligible entropy of mixing).

CLIC-GEEC: Clathrin-independent carrier/GPI-AP-enriched early endosomal compartment.

of extracellular fluid containing soluble or dispersed material; this process leads to the formation of rather large vesicular bodies (macropinosomes, 0.5 – 5 μm), which in leukocytes and renal cells have an endolysosomal evolution; in other cells, less engaged in degradative or antigen-presenting processes, the macropinosomes fuse back with the cell membrane, probably reorganizing it [20]. The protrusions are generally produced in regions where the cell membrane is highly ruffled [21] and, although the precise mechanism of their formation and closure is not yet perfectly understood, it is known that the process is highly dependent on F-actin polymerization, in a fashion similar to phagocytosis [9].

Macropinocytosis is involved, generally in a non-exclusive fashion (e.g., in parallel with clathrin-mediated endocytosis), in the internalization of several antigen-presenting objects, such as viruses [22], cell-penetrating peptides [23] and also large macromolecules, such as DNA both in naked or in complexed form [24].

2.3 Circular dorsal ruffles

Circular dorsal ruffles (also called 'dorsal waves') are a relatively recently discovered mechanism used for the internalization of rather large volumes of fluids through the F-actin-dependent formation of tubular structures on the dorsal surface of adhering cells [25,26]. These structures are known to be formed in response to the activation of receptor tyrosine kinases on binding with growth factors, such as epidermal growth factor (EGF) or platelet-derived growth factor; although morphologically different in its first phase, this endocytic mechanism is substantially analogous to macropinocytosis, as the tubular structures evolve in micropinosomes.

2.4 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the most studied mechanism of receptor-mediated internalization and probably also the most widely used by virtually all cell types for the internalization of macromolecular and nanosized materials.

Possibly the best known receptors adopting this mechanism are transferrin [27], the low-density lipoprotein (LDL) receptor [27,28], the epidermal growth factor receptor (EGFR, a receptor tyrosine kinase) [29] and the β2-adrenoceptors (G-protein coupled receptors) [30]. There is compelling evidence that the endocytic machinery often requires cooperation between different receptors, although not necessarily all present in a bound form: for example, unbound EGFR can be transactivated by the bound β2-adrenoceptor [31].

In the most frequent case, clathrin trimeric structures (triskelions) multimerize to yield highly curved invaginations termed clathrin-coated pits; larger and flatter clathrin-coated endocytic structures (plaques) have also been found, although only at the interface with adhesive substrates, not on the free sites of cell membranes [32]. Both the highly curved pits and the flatter plaques evolve into clathrin-coated vesicles after pinching and scission of the cell membrane by dynamin (**Figure 2**).

Clathrin is eventually recycled to the cell membrane, uncoating the vesicles that evolve into early endosomes; early endosomes then undergo acidification and fuse with other vesicular structures mostly originating from the Golgi apparatus, giving rise to late endosomes where most ligand–receptor complexes dissociate, interrupting signaling cascades that started at the cell surface [33]. Late endosomes are also referred to as multivesicular bodies (MVBs) owing to the frequent presence of further internal vesicular structures. The ligands or the residual ligand–receptor complexes are then sorted to different cellular compartments or remain in the endosome during its fusion with lysosomes, where the local environmental conditions (proteolytic enzymes, low pH) eventually lead to the degradation of the internalized structures.

2.5 Caveolae-mediated endocytosis

Caveolae-mediated endocytosis is possibly the most common clathrin-independent mechanism of receptor-mediated endocytosis. Caveolae are small (60 – 80 nm), flask-shaped invaginations in the plasma membrane that are particularly abundant in fibroblasts, endothelial cells, smooth muscle cells and adipocytes [34]. The main membrane proteins found in caveolae are named caveolins: caveolin-1 and -2 are found in non-muscle cells, whereas caveolin-3 is typical for both smooth and skeletal muscle cells. Despite its colocalization with caveolin-1 [35], the role of caveolin-2 appears to be rather ancillary: caveolae can be formed in the absence of caveolin-2, but not in the absence of caveolin-1. Caveolins form oligomeric scaffolds tightly bound to the invaginating portion of the membrane, which can be described as a lipid raft. As most lipid rafts are on cell membranes, these too are particularly rich in cholesterol and sphingolipids. It is important to note that caveolin-1 is not found only in caveolae, but also in other membrane-related complex structures ('microdomains'), which are often, but not necessarily, associated to lipid rafts [36].

Differently from clathrin pits, caveola formation takes place in the cytoplasm, involving both endoplasmic reticulum (ER, where caveolins are synthesized) and Golgi (where early caveolae are assembled); caveolae end up localizing on the cell membrane only on complete maturation [35]. On the cell surface, it is not uncommon for caveolae to form interconnected networks with a proposed cubic geometry [37], as is frequently observed in adipocytes and sometimes also in fibroblasts. The detachment of caveolae from the cell surface is a dynamin-mediated process [38]; the budded caveolae then fuse with early endosomes [39] and also, differently from what happens for clathrin-coated pits, with caveosomes [40], where they do not undergo acidification. Typically, instead of being channeled to lysosomes, the early endosomes and caveosomes formed by the budded caveolae are directed to the Golgi complex or to their discharge through transcytosis. This picture, however, is somehow disputed, in particular regarding the occurrence of fusion events: there are conflicting reports about the intracellular fate of the caveolae and they may arise because of the interpretation of still membrane-bound (multicaveolar) structures as cytoplasmic ones [41].

The internalization through caveolin-mediated endocytosis opens the way to the possibility of avoiding the aggressive lysosomal compartment and also, if caveosomes are involved, any form of acidification; this suggests that the cell penetration of several pathogens and/or toxins, such as SV40 viruses [42] or cholera toxins [43], takes place through this mechanism. Receptor-dependent caveolin-mediated endocytosis appears to be relevant also for the internalization of cell adhesive (integrin-binding) cues of the extracellular matrix, as the internalization of both fibronectin [44] and integrins [45] has been shown to utilize (also) this mechanism. It is noteworthy that recent evidence has pointed out that caveolae appear also to perform some non-endocytic functions (from mechanosensing to NO and calcium signaling), whereas their stability and low mobility appear to be contrasting, with a predominantly endocytic role, at least in the absence of a specific activation [41]. As the colocalization of a drug carrier within caveolae does not therefore imply an effective and rapid release in the cytoplasm, it is possibly more appropriate to consider caveolae-dependent endocytosis as a viable mechanism for targeted drug delivery only in the presence of specific, well-known groups, such as the hydrophobic folic acid (vitamin B_9) or cholesterol, which are known to stimulate their mobility.

2.6 Clathrin- and caveolin-independent endocytosis

Clathrin- and caveolin-independent endocytosis is a generic name for defining endocytic mechanisms of rather recent discovery. Hereafter, the probably most significant ones are listed.

(1) *Flotillin-dependent endocytosis*. This mechanism bears strong analogies to caveolin-dependent endocytosis: flottilin-1 and -2 are two co-assembling membrane proteins associated to lipid rafts (through palmitoylation, similarly to caveolins) that are capable of generating cell membrane microdomains budding into the

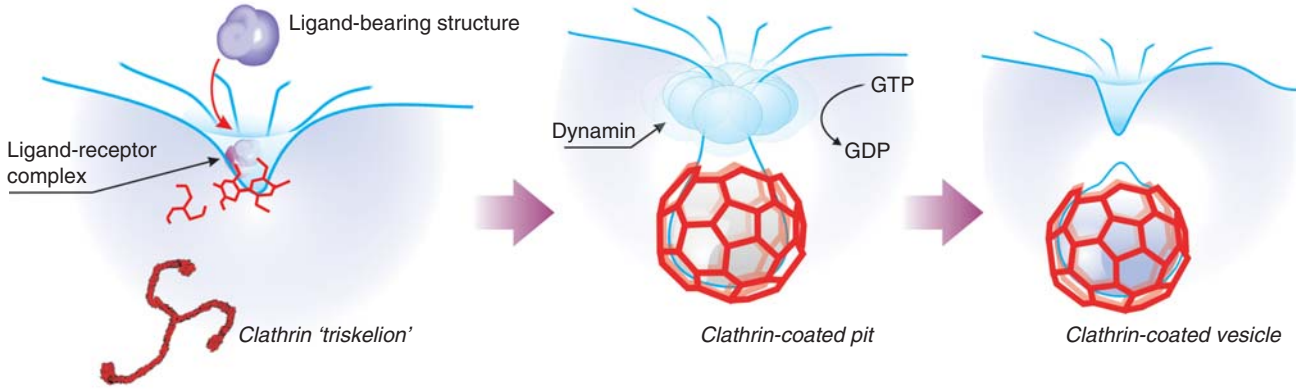

Figure 2. Sketch of the time evolution of clathin-mediated endocytic processes: clathrin triskelia (assemblies formed by three clathrin light chains and three clathrin heavy chains) are recruited at the site of a (multiple) ligand–receptor binding. The triskelia multimerize in highly curved, membrane-bound structures, which determine an invagination of the membrane. The resulting clathrin-coated pit is then detached from the membrane by the GTP-fuelled contraction of dynamin and evolves into a clathrin-coated cytoplasmic vesicle; the clathrin coat will then be lost and clathrin recycled to the cell surface.

cytoplasm [46,47]. It has been shown that the internalization of glycosylphosphatidylinositol-anchored proteins (GPI-APs) may use flotillin-dependent endocytosis [48], as well as caveolins or other mechanisms (see point 2). This class of proteins is extremely important in targeted drug delivery: several receptors, including the two most important ones for folic acid [49] (see Section 3.7), belong to this class.

(2) *Clathrin-independent carrier/GPI-AP-enriched early endosomal compartment.* This pathway is also known as cdc42-dependent uptake, where cdc42 is the acronym for the cell division control protein 42 homolog, a G protein with GTPase activity, involved in the control of many phases of the cell cycle. This form of endocytosis is based on the formation of elongated invaginations (tubules), which evolve into tubular early endosomes. Despite the morphological difference and the apparent independence of dynamin (necessary for the detachment of caveolae or clathrin-coated vesicles from the cell membrane), clathrin-independent carrier/GPI-AP-enriched early endosomal compartment (CLIC/GEEC) has a significant overlap with caveolin-1-mediated uptake: the activity of cdc42 is known to be modulated by caveolin-1 and caveolin-1 and is also found in the CLIC/GEEC tubules, although it is not necessary for their formation [19]. Similarly to flotillin- and caveolin-mediated uptake, CLIC/GEEC is also associated with the formation of lipid rafts, as it is strongly affected by depletion in sphingolipids [50]. As suggested by the name, CLIC/GEEC is possibly the main contributor to the internalization of GPI-APs, although not the exclusive one: they may also be internalized through caveolin- and flotillin-mediated uptake [48]; it has also been shown that CLIC/GEEC inhibition determines a switch to a clathrin-mediated internalization of GPI-APs, although it is not clear whether this pathway leads to the same intracellular destinations [51]. CLIC/GEEC is the most typical mechanism of internalization also for fluid phase markers such as dextran [52], and for several toxins, such as cholera toxin B, ricin and the *H. pylori* vacuolating toxin [53]. The only marker marker of CLIC/GEEC found so far is a GTPase-activating protein, GRAF1 [54].

(3) *Interleukin 2 receptor β pathway, or RhoA-dependent uptake.* RhoA (Ras homolog gene family, member A) is a GTPase involved in the regulation of actin polymerization, and its inhibition has been shown to switch off the internalization of a few receptors. This mechanism is dependent on sphingolipids for the formation of invaginations [50] and on dynamin for vesicle budding, and leads to an endolysosomal evolution. It has been demonstrated to be active in the internalization of a few specific receptors after their ligation: interleukin 2 receptor β (IL-2Rβ) [55], whose internalization was recognized to be dependent on the presence of lipid rafts before an *ad hoc* mechanism was postulated [56], and the γc cytokine receptor [57], which is part of the same family of cytokine receptors. The high-affinity receptor FcεRI, which binds to the least common of immunoglobulins, that is, the IgEs, was originally thought to be internalized through clathrin-mediated uptake; indeed, it has been shown to colocalize with clathrin-coated pits, and also to depend on RhoA and not on clathrin for internalization [58]. It is noteworthy that other immunoglobulins, such as the most common IgAs, are internalized through clathrin-mediated mechanisms [59].

(4) *Arf6 (ADP-ribosylation factor 6)-dependent uptake.* This more elusive endocytic mechanism has been invoked in the

internalization of several proteins, including class I major histocompatibility complex molecules (MHC I), β_1 integrin, E-cadherin and some GPI-APs [53,60]. The mechanism appears to be dynamin-independent and possibly to share some features with CLIC/GEEC. However, cell- and species-specific features [53] and possible overlaps with other endocytic mechanisms, such as clathrin-mediated uptake [61-63], have hindered a clear elucidation of this mechanism so far.

3. Internalization receptors used in targeted drug delivery

The targeting of nanocarriers for biologically selective and intracellular delivery of encapsulated payloads has focused on only a handful of cell surface receptors. As will become apparent from this part of the review, most of these receptors use various forms of clathrin-mediated uptake.

3.1 Transferrin receptor (clathrin-mediated uptake)

The transferrin receptor (TfR), a homodimeric membrane receptor of 90 kDa per subunit, is responsible for the active uptake of transferrins (transferrin, lactotransferrin and melanotransferrin). These non-heme glycoproteins transport iron (Fe^{3+}) from sites of intake, for example the oral mucosa, to blood and then to cells and tissues. The most important member of this family is indeed transferrin and the uptake of its iron-bound form (holotransferrin, as opposed to the free form, which is called apotransferrin) is regulated by two receptors that appear to have complementary functions: TfR1 is supposedly related to 'homeostatic' and TfR2 to 'overload' iron uptake [64]. Following transferrin endosomal uptake and release of transferrin-bound iron inside the cell, recycling of ligand–receptor complex occurs [65]. The overexpression of TfR1 is a common signature of most human cancers, which possibly require extra iron supply for their rapid growth [66]. As a result, transferrin conjugation to nanocarriers has become a widespread approach for specific cellular uptake of drugs/genes [67,68]. Transferrin endocytc uptake is generally a clathrin-dependent mechanism [64]; although most of its nanoconjugates have not been investigated in great detail, it is expected that this mechanism of uptake applies also to them. Transferrin uptake, however, has raised much interest not only for possible applications in intracellular delivery, but also for its use in overcoming cellular barriers through a transcytotic pathway. Particular attention has been paid to the case of the blood–brain barrier (BBB), and the use of TfR to avoid at least partially the action of efflux transporters at the BBB is now one of the most investigated avenues to facilitate the brain delivery of active principals [69]. TfRs are expressed on the apical membrane, that is, the blood-contacting side, of the BBB endothelial cells [70], and are continuously internalized after binding to holotransferrin [71]; the transcytosis of this endocyted material to the brain parenchyma is the main mechanism of iron transport to the brain [72]. During the endosomal permanence holotransferrin dissociates into Fe III, which is reduced to Fe II and rapidly exocyted, and halotransferrin. The transcytosis of the latter, however, has been a source of controversy, as some authors have reportedly failed to observe it [70] or suggested this to be a rather minor mechanism compared with the whole iron transcytosis [73]. On the other hand, overwhelming evidence has supported the occurrence of transcytosis for both transferrin, the monoclonal antibody for the TfR (OX26) [74], and the OX26 conjugates with drug molecules [75] and nanocarriers, such as liposomes [76]. The presence of initial conflicting evidence was possibly caused by a lower transcytosis activity of iodinated (radiolabeled) transferrin that could be completely inhibited by the presence of endogenous transferrin [77]. A few recent examples of TfR targeting for intracellular delivery follow.

Transferrin-conjugated poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles loaded with paclitaxel have displayed greater inhibitory effects on cell growth than free paclitaxel in MCF-7 and MCF-7/Adr cells expressing multi-drug resistance [78]. It was demonstrated that the sustained intracellular drug retention mediated by Tf-conjugated nanoparticles can partially overcome drug resistance. In another study, a transferrin-conjugated liposomal formulation allowed a significant increase in the transfection efficacy of p53 (a tumor-inhibiting protein), resulting in the sensitization of the transfected cancer cells/xenografts to ionizing radiation [79].

A multifunctional nanoparticle system based on cyclodextrin-containing polycations was designed by Barlett and Davis [67]. The inclusion complexes formed between adamantane-containing poly(ethylene glycol) (AD-PEG) and the β-cyclodextrin residues enabled the modular attachment of steric stabilizers and targeting ligands (AD-PEG-transferrin). Competitive uptake experiments showed that the transferrin-targeted particles displayed enhanced affinity for the transferrin receptor through avidity effects (multiligand binding), while delivery of pDNA and siRNA was proved by luciferase reporter protein expression and knockdown, respectively [67].

Kompella *et al.* investigated the effect of coating 20 nm polystyrene nanoparticles with peptide or protein ligands for cell surface receptors-enhanced ocular delivery and reported that deslorelin and transferrin conjugation enhanced corneal epithelial uptake of nanoparticles by 4.5- and 3.8-fold at 60 min, respectively, in an *ex vivo* bovine eye model [80].

3.2 Receptor tyrosine kinases (EGFR) (clathrin-mediated uptake)

The epidermal growth factor receptor is one of most investigated receptor tyrosine kinases, with a high binding affinity not only for EGF, but also for several other ligands. Within the ErbB family of receptor tyrosine kinases EFGR (also known as ErbB1) and HER2 (also known as ErbB2) have a specific relevance in the pathogenesis of cancer [81]: the over-presentation of EGFR and HER2 in several tumors has

been shown to be linked to their reduced endocytic downregulation, which appears to have an oncogenic effect [81]. To counter this, antibodies for HER2 and EGFR have been developed to force their clathrin-mediated endocytosis and degradation: trastuzumab (also known as herceptin, a humanized monoclonal antibody for HER2) and cetuximab (also known as erbitux, a chimeric mouse/human monoclonal antibody for EFGR) are at the basis of advanced chemotherapy treatments for early breast cancer and for colorectal and head and neck cancer, respectively [82,83].

Owing to the over-presentation/overexpression of EFGR in tumors, this has also become a potential target for promoting a preferential uptake of nanomaterials in tumoral cells [84-88]. *Inter alia,* this strategy may combine the benefits of a targeted chemotherapy with those of an increased endocytic downregulation of a possibly oncogenic receptor (see above). EGFR is mostly internalized in a clathrin-dependent fashion, thus endocytosis will occur along the endolysosomal pathway, which should determine an increased exposure of the payload to an acidic environment. However, it has recently been reported that the clathrin-mediated EGFR uptake mostly leads to receptor recycling rather than to its degradation, which on the contrary occurs through a non-clathrin-dependent pathway [89]. This finding provides, therefore, a caveat: the adoption of a clathrin-mediated mechanism may not necessarily lead to endosomal accumulation of an EGF-decorated nanocarrier. A few examples of EGFR targeting follow.

Biotinylated EGF has been coupled to the surface of neutravidin-displaying gelatine nanoparticles to achieve EGFR-mediated endocytosis into lung cancer cells. Flow cytometry revealed higher internalization of the nanoparticles in A549 adenocarcinoma cells, overexpressing EGFR, than in normal lung cells (HFL1). Confocal microscopy demonstrated lysosomal entrapment in A549. When delivered by aerosol administration to the lung of severe combined immunodeficiency (SCID) mice, the nanoparticles accumulated in the tumor, indicating their *in vivo* targeting capacity [85].

Rapamycin-loaded PLGA nanoparticles were surface-conjugated with antibodies to EGFR using carbodiimide/*N*-hydroxysuccinimide (NHS) chemistry. MTT assays revealed superior antiproliferative activity of EGFR antibody-conjugated nanoparticles over unconjugated ones and over free rapamycin, owing to higher cellular uptake in MCF7 malignant breast cancer cells. Flow cytometry further confirmed cell cycle arrest and cellular apoptosis, whereas western blotting revealed the involvement of a cytoplasmic protein in activating the programmed cell death pathway [88].

Dual-ligand liposomes simultaneously targeting both folate receptors (see later) and the EGFR were designed by Saul *et al.* [86]. The authors hypothesized that a low number of dual-targeted carriers would be toxic to tumor cells but spare the healthy ones (off-target cells) that express only one or none of the targeted receptors. To test this hypothesis using doxorubicin as a model drug, cytotoxicity was determined using KB cells that express both FRs and EGFR and off-target control cells in which one or both receptors were blocked. The data demonstrated a significant enhancement of selectivity, determined through the LC50 ratios for single- and dual-ligand formulations, showing that dual-ligand liposomes were capable of improving selectivity over single-ligand formulations.

3.3 Lectins (mostly clathrin-mediated uptake)

Lectins are saccharide-binding membrane proteins, which can bind to sugars either in a free form or supported on other membranes, for example, in the form of glycolipids or glycoproteins.

3.3.1 *Lectins as targeted receptors*

Receptors of the mannose receptor family (a subgroup of C-type lectins) are among the most studied lectins; reportedly they all undergo endocytosis in clathin-coated pits. Most of them allow, at least in principle, some form of targeting, as they are predominantly (although not exclusively) expressed in a limited number of cell lines: the mannose receptor in macrophages, DEC-205 in dendritic cells, Endo180 in fibroblasts, activated chondrocytes, stromal cells and macrophages [90]; DC-SIGN is another C-type lectin typically expressed by dendritic cells. Another member of this family, the M-type phospholipase A2 receptor (PLA2R), shares a similar endocytic mechanism, but does not show preferential expression in any cell type. Other important lectin receptors include the asialoglycoprotein (ASGP) receptor, which is mostly expressed by hepatocytes and binds galactose or *N*-acetylgalactosamine residues [91,92], and the mannose 6-phosphate receptor, which is a particularly important cargo for intracellular sorting of endocytic material [93]. Both receptors are internalized through clathrin-mediated uptake. Among all the above receptors, the mannose one is the most popular target [94].

It is noteworthy that clustering of ligands [95] and/or receptors [96] can significantly potentiate the strength of the saccharide/lectin interactions; therefore, knowledge and control over the density of low-molecular-mass saccharidic ligands along the chain of a polymer carrier or on the surface of a nanocarrier is essential for determining the overall avidity of the delivery vehicle and also for overcoming the low affinity of many of these interactions. A few examples of nanocarriers designed to use lectin receptor-based endocytic pathways follow.

The conjugation of dimannoside groups has allowed efficient delivery of melanoma antigens to dendritic cells, whereas lactoside conjugates were inefficient; this selectivity was attributed to the selective binding of mannosides to mannose receptor and DC-SIGN [97].

Chitosan oligomer polyplexes for gene delivery were designed to target the asialoglycoprotein receptor by introducing a specific trisaccharide branch. Live-cell confocal microscopy showed improved cellular uptake in HEK 293 cells (11-fold) for the trisaccharide-substituted (TCO) polyplexes compared with the linear chitosan oligomers. Higher gene expression levels in the human liver hepatocyte (HepG2) cells (10-fold) were observed with the TCO polyplexes than those

mediated by polyethyleneimine. A similar improvement was obtained in a human bronchial epithelial cell line (16HBE14o-) overexpressing lectin receptors, confirming lectin-mediated uptake. Finally, *in vivo* studies showed a four-fold higher luciferase gene expression with the TCO than with the corresponding linear chitosan oligomers 24 h after lung administration to mice [98].

3.3.2 *Lectins as targeting ligands*

In this approach, nanocarriers functionalized with lectins have been used to target possibly overexpressed sugar residues on the surface of specific, often epithelial, cell types [99]. The most popular lectin used for this purpose is wheat-germ agglutinin (WGA), which essentially targets sialic acid and *N*-acetylglucosamine residues, although others have been used too, for example, peanut agglutinin (→galactosamine) [100,101], or *Dolichos biflorus* agglutinin (→*N*-acetylgalactosamine) [100]. Very little attention has been devoted to the mechanistic study of their internalization behavior; one of the few studies available reportedly mentions the involvement of both clathrin- and caveolin-mediated uptake in the internalization of WGA-functionalized nanoparticles [102]. A few examples of the use of lectin-functionalized nanocarriers follow.

Russell-Jones *et al.* used WGA, concanavalin A (ConA), which binds to mannose, and LTB, the binding subunit of *Escherichia coli* heat labile toxin, LT, which binds to GM-1 ganglioside and galactose, to coat commercially available fluorescent nanoparticles in a range of sizes from 50 to 500 nm. The uptake of nanoparticles was directly proportional to the amount of targeting agent attached. Lectin-mediated nanoparticle uptake was found to be readily inhibited by co-administration of free lectin, or of the specific sugar or ganglioside to which the lectin was known to bind [103]. WGA-conjugated PLGA nanoparticles incorporating thymopentin (TP5, a potent immunostimulant) showed an improved intestinal absorption of TP5 resulting from specific bioadhesion on the membranes of the gastrointestinal tract cells [104].

Lectins have also been used as ligands to enhance nasal adsorption of nanoparticles [105]. PEG-PLGA nanoparticles were functionalized with thiolated WGA, aiming at the abundant presence of *N*-acetyl-D-glucosamine and sialic acid residues on the cells in the nasal cavity. WGA induced a strong mucoadhesion and triggered or facilitated the active transport of nanoparticles through the nasal mucosa; even more interestingly, its presence increased the access of coumarin-loaded nanoparticles to the blood circulation and to the brain.

3.4 Other receptors for clathrin-mediated uptake

Riboflavin (vitamin B_2) appears to show a clathrin-dependent uptake of its receptor on binding [106]; it has been reported (although rather episodically) that the riboflavin carrier protein is overexpressed in some cancers [107] and as a result riboflavin has been evaluated with some initially positive results as a possible targeting ligand [108]. This suggestion, however, now appears to have been abandoned.

Cobalamin (vitamin B_{12}) binds cubilin, also known as receptor for B-12-intrinsic factor (IF) complex, and the complex is internalized through the LDL receptor machinery by means of a clathrin-mediated mechanism [109,110]. As an example, this pathway has been utilized to internalize and transport vitamin B_{12}-modifed nanoparticles in Caco-2 cells. The results showed that both IF-independent and IF-dependent pathways contributed to nanoparticle uptake and transport in caco-2 cells and that both were dependent on the density of cobalamin on the surface of nanoparticles, although in a slightly different fashion [111].

3.5 Cell adhesion molecule receptors (mixed dependence)

Receptors of cell adhesion molecules (CAMs) are, most commonly, endocyted by means of a clathrin-dependent mechanism, both when in a soluble form or in a cell-attached one. This applies, for example, to the internalization of cadherin-containing complexes composing adherent and tight junctions during phenomena of loss of epithelial phenotype or increase of vascular permeability [112,113]. To the authors' knowledge, however, cadherins have not been systematically targeted as a portal for intracellular delivery.

The case of integrins is different: their internalization mechanism appears to depend on the composition of the α,β-heterodimeric receptor the unit appears to be controlling [114]; for example, a clathrin-mediated mechanism has been shown to govern the uptake of $\alpha_v\beta_5$ and $\alpha_v\beta_6$ [115,116] and of several β_1-containing heterodimers [117]; on the other hand, other integrins such as $\alpha_2\beta_1$ appear to be associated with caveolins [118]. It has also been hypothesized that the same heterodimers may follow different internalization paths depending on their localization on the cell membrane [119].

Integrins are an excellent target and they have been widely investigated for the purpose of targeted drug delivery: besides being essential in determining and modulating cell adhesion, several members of this family are upregulated in numerous tumors, where they play an important role in angiogenesis and metastasis [120,121]. The aminoacidic sequence arginine-glycine-aspartic acid (RGD), present in many extracellular matrix proteins, is very popular for conferring cell adhesion to tissue-engineered constructs [122,123]; this sequence has been shown to bind preferentially to integrin $\alpha_v\beta_3$; this integrin pair is frequently overexpressed on endothelial cells in tumor neovasculature [124], which, owing to their accessibility, are considered as a major target for therapeutic intervention. A few examples of the use of RGD-displaying nanocarriers follow.

It has been shown that the antitumor efficacy of doxorubicin-loaded liposomes was improved by targeting them to the vasculature of colon cancer using RGD peptides in a xenograft mouse [125]. Crosslinked carbohydrate-based (inulin multimethacrylate) nanoparticles have been designed to incorporate the same drug (doxorubicin), displaying a PEG-tethered cyclic RGD as the targeting peptide.

Pharmacokinetics and biodistribution studies revealed decreasing drug concentrations over time in the heart, lung, kidney and plasma and accumulating drug concentrations in the liver, spleen and tumor [126].

Liu *et al.* synthesized RGD-modified poly(lactic acid-*co*-L-lysine) (PLA-PLL-RGD) and used it to prepare nanoparticles loaded with the antitumoral agent mitoxantrone [127], which demonstrated significantly higher antitumor efficacy than free mitoxantrone in hepatoma-bearing and breast carcinoma-bearing mice.

In vitro studies on human umbilical vein endothelial cells (HUVEC) have demonstrated that paclitaxel-loaded PEGylated PLGA nanoparticles have an enhanced cellular uptake mediated by $\alpha_v\beta_3$ integrin when grafted with the RGD peptide or RGD-peptidomimetic (RGDp) [128]. *In vivo* immunohistochemistry on syngeneic transplantable liver tumor (TLT)-bearing mice demonstrated that RGD-displaying nanoparticles targeted the tumor endothelium more extensively than control ones. When the nanoparticles were loaded with paclitaxel, this targeting was associated with delayed tumor growth and higher survival rate for mice [128]. The replacement of RGD with RGD-mimetic peptides allowed enhanced association and binding to HUVEC but did not result in an increased antitumor efficacy *in vivo;* this result may also be ascribed to the presence of multiple internalization mechanisms.

3.6 Cell penetrating peptides (mixed dependance)

Cell penetrating peptides (CPPs), also known as protein transduction domains, are up to 30 amino acid-long peptides derived from viral, insect or mammalian proteins endowed with membrane translocation properties. CPPs have the ability to cross the plasma membrane of mammalian cells; this may allow the intracellular delivery of membrane-impermeable CPP-conjugated therapeutic payloads, such as peptides, proteins, oligonucleotides, plasmids and even nanometer-sized particles. In general, CPPs do not share a common amino acid sequence. Some of them have a cationic character, which generally derives from the presence of arginine groups; typical examples of these guanidinium-rich transporters [129] are the TAT peptide, that is, the 9 aminoacidic sequence RKKRRQRRR corresponding to positions 49 – 56 in the HIV TAT protein [130], TAT-derived peptides [131] and peptoids [132], oligoarginines (R_6 – R_{20}) [133] and penetratin (RQIKIWFQNRRMKWKK) [134,135].

Despite their widespread use, there has been surprising little consensus regarding the exact mechanism of internalization. Earlier studies suggested a passive, direct transfer through the plasma membrane, which was based on electrostatic interactions and hydrogen bonding and was neither energy-dependent [136] nor sensitive to the presence of endocytic inhibitors [137]. For example, penetratin and Tat peptides were proposed to form inverted micellar structures after positively charged peptides interact with negatively charged phospholipids of cell membrane, directly transferring a payload through the lipid bilayer [138]. A more careful re-evaluation of the CPPs' action has shown, however, that artefacts during cell fixation experiments severely affect the peptide distribution and that, indeed, the CPP internalization should be receptor-dependent [139]. Still, although the mechanism of internalization of these cargos appears to be endocytic in nature, evidence has shown the contemporaneous occurrence of clathrin-mediated, caveolin-mediated and macropinocytic uptake [129,140], indicating therefore a rather mixed and until now undefined mechanism. A few examples of the use of nanocarriers conjugated to cationic CPPs follow.

A high density of octaarginine (R_8) on the surface of liposomes stimulated macropinocytosis, and improved transfection efficiency by avoiding lysosomal degradation [141]. The decoration with octaarginine of a DNA-containing multifunctional envelope-type nanodevice (MEND) allowed its internalization by means of macropinocytosis, bypassing lysosomal degradation. The MEND-mediated transfection efficiency of luciferase expression was comparable to adenovirus-mediated transfection, with a lower associated cytotoxicity. When a bone morphogenetic protein (BMP) type IA receptor (ca*Bmpr1a*) gene was loaded in MENDs topically applied to mouse hair follicles, significant hair growth was observed [142].

The decoration of gold nanoparticles with different TAT peptides has shown that a highly cationic TAT peptide allows nuclear delivery of the particles [143]. The importance of the overall charge for nuclear delivery is indeed well known and had already been stated in several previous reports: see, for example, Zelphati and Szoka [144], who demonstrated that polyplexes require a positive charge for oligonucleotide delivery to the nucleus.

Park *et al.* [145] have recently used low-molecular-mass protamines that are structurally similar to the HIV–TAT protein transduction peptide. The protamines formed complexes with DNA with size > 100 nm and a positive zeta-potential (+30 mV). These complexes demonstrated similar transcellular localization behavior and kinetics to DNA–TAT complexes, and rapidly transferred DNA into the nucleus.

Another large grouping of CPPs owes their penetration to their amphipaticity, which derives from the presence of spatially distinct hydrophobic and hydrophilic portions, possibly adapted in an α-helical structure [146]. Examples of such structures are transportan (a chimeric structure derived from wasp venom, GWTLNSAGYLLGKINLKALAALAKKIL) [147], VP22 and PFVYLI [148]. Penetratin, in addition to being cationic, is amphipatic too. Their internalization mechanism appears to be based sometimes on direct interactions with the lipid bilayers [147]; this mechanism makes them less suitable for use as a surface ligand on a nanocarrier, where the solubility in the membrane and the overall amphipaticity would be dictated rather by the rest of the carrier structure than by the ligand itself. However, endocytic mechanisms are possible too; at least for transportan, flotillin-mediated

uptake has been excluded in favor of a caveolin-mediated endocytosis [149].

3.7 Folate receptors (caveolin-mediated and other mechanisms)

Folic acid (vitamin B_9) derivatives are internalized through the action of a few different receptors: the predominant transporter in homeostatic conditions is the reduced folate carrier (RFC), which binds to the reduced form of vitamin B_9 but not to folic acid, and has a relatively low affinity (dissociation constant in the micromolar range) but a high capacity. The folate receptor (FR), on the contrary, has a high affinity (dissociation constant in the sub-nanomolar range) for folic acid and for several its derivatives; under the name of FR one includes several different isoforms: the most prominent ones are FR-α and FR-β, which are membrane-associated GPI-APs [150] characterized by high affinity towards folic acid derivatives, although the two isoforms interact with different strengths with the same compounds. FR-α is normally found in epithelial cells, but is significantly overexpressed in several tumors: primarily in the adenocarcinomas of ovary, uterus and pituitary gland, in mesotheliomas and in ependymal brain tumors, and also, but less reproducibly, in colon, breast and kidney tumors. Although variable in intensity, FR-α overexpression is believed to be common to over one-third of human cancers and it may be related to an increased demand for the coenzyme folic acid, which is necessary for the synthesis of amino acids and nucleic acids. FR-β, on the other hand, is normally present during myelopoiesis (the formation of granulocytes, monocytes and mast cells) and is constitutively overexpressed in activated macrophages [151] and in leukemia [152].

Folate conjugation to a variety of low-molecular-mass compounds as well as nanomaterials [153-156] has been demonstrated to promote their intracellular uptake through folate-mediated endocytosis. In general, it could be hypothesized that folate conjugates are internalized preferentially through a clathrin-independent pathway, as they are based on the use of a GPI-AP as a receptor. However, detailed investigations of these mechanisms (e.g., caveolin-mediated versus flotillin-mediated, Arf6-mediated or CLIC/GEEC) have seldom provided unequivocal results. A few indicative examples follow.

The enhanced endocytic uptake of trimethylchitosan/pDNA complexes following conjugation with folate was inhibited by free folate (1 mM); the presence of folate also increased the transfection efficiency up to 1.6-fold in KB cells and SKOV3 cells (FRs-positive cell lines), whereas it had no effect in A549 cells and NIH/3T3 cells (FRs-negative cell lines) [157]. Similarly, Esmaeili *et al.* reported increased cytotoxicity after 96 h incubation and enhanced cellular uptake of docetaxel-containing nanoparticles based on a poly(lactide-*co*-glycolide)-*b*-poly(ethylene glycol)-folate (PLGA-PEG-FOL) conjugate; their rapid internalization in folate receptor-bearing cancer cells appeared to bypass their multi-drug efflux pumps [158]. In another study, folate-decorated poly(lactide)-vitamin E TPGS nanoparticles were used to deliver paclitaxel, showing enhcanced cytotoxicity in FR-rich cells such as MCF-7 and C6 cancer cells [159]. Wang *et al.* [160] proposed a combined targeting based on folate to promote active internalization and Pluronic to overcome multi-drug resistance. The results showed that the Pluronic micelles decorated with folate exerted a synergistic effect on the reversal of multi-drug resistance in MCF-7/ADR tumor cells and were useful for treatment of multi-drug resistance in solid tumors.

Recently, an elegant system was developed utilizing cysteine-cleavable phospholipid-PEG conjugates that mask folate ligands while in circulation, thus enabling passive targeting of liposomes to solid tumors through the EPR effect. Following extravasation at the tumor site, the conjugates are deprotected, allowing the exposure of folate for targeting FR-overexpressing cells [161].

The number of drug nanocarriers developed for FR-mediated internalization is vast, and the above examples are just indicative of the current research activity in this area [162,163].

4. The endocytic detective's tools

The endocytic uptake of nanocarriers is generally studied through a mixture of qualitative and quantitative techniques. Different forms of microscopy allow for the qualitative visualization of the intracellular localization of nanocarriers, most commonly confocal microscopy, for example using fluorescently tagged nanocarriers or utilizing cargo molecules (e.g., siRNA, antisense DNA) that inhibit the fluorescence of cells [164], but also electron microscopy, above all for metal-containing nanocarriers. A list of common fluorescent markers/used for endocytosis visualization in confocal microscopy is provided in **Table 1**.

The quantification of the cellular uptake is most often based on flow cytometry [165-169], microfluorimetry [111,170-172], or quantitative extraction of the nanoparticles from the cells [173-175], and it is generally coupled to the assessment of its mechanism through the use of more or less selective molecules or environmental conditions that inhibit individual endocytic pathways.

A list of the most commonly used inhibitors is provided in **Table 2**. Although simple and readily achievable, this approach is rather crude: for example, most inhibitors have a broad spectrum and may knock down more than one endocytic mechanism, as happens for the inhibition of actin polymerization, which knocks down all processes requiring cytoskeletal reorganization. Also, inhibitors are not known for the more elusive mechanisms and, finally, secondary mechanisms may compensate the inhibition of a primary one. This happens, for example, for the clathrin-mediated internalization of the detergent-resistant membrane-associated IL-2 receptor (a GPI-AP) when CLIC/GEEC is inhibited (by the inhibition of cdc42) [51]. However, the secondary

Table 1. Fluorescent probes commonly used for studying endocytic processes.

Marker	Application/relevance	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	Ref.
Dextran 70,000, Texas red labeled	Macropinocytosis	595	615	[169]
Lucifer yellow	Internalized via non-coated vesicles/fluid phase marker	428	535	[181]
Chlolera Toxin, subunit B, Alexa 594 labeled	Caveolae-mediated endocytosis	589	616	[182]
Transferrin, Alexa 594 labeled	Clathrin-mediated endocytosis	589	617	[183]
Lysotracker red-DND99	Late endosomes and lysosomes	577	590	[184,185]
Phalloidin, Texas red labeled	Actin filaments	595	615	[186]
DAPI	Nuclear stain	358	461	[187,188]
Concanavalin A, Alexa Fluor 594®	Cell surface glycoprotein	590	617	[189]
FITC (or others)	Fluorescent label for nanoparticles	490	518	[172,190-192]
Trypan blue	Extracellular probe (quench non-internalized FITC-labeled nanoparticle fluorescent signals)	525	–	[193-196]

mechanism may not necessarily be active when the first one is not inhibited. Last but not least, several inhibitors have been shown to affect cell viability detrimentally under the conditions usually adopted for endocytosis studies [176].

The use of genetically engineered cells would allow a more selective intervention on individual endocytic mechanisms; however, this structural deficiency may also impair other important cellular functions, as has been demonstrated for clathrin-deficient yeast cells [177,178].

More interestingly, siRNA has been used recently for a reversible inhibition of the production of proteins that are involved in the different endocytic processes: this approach allows for the growth of perfectly functional cells, allowing therefore for a more precise and reliable analysis of the endocytic mechanisms. After the seminal work of Huang *et al.,* where SiRNA was used to knock down the expression of clathrin heavy chains and of the transferring and EGF receptors [179], several other studies have shown the viability of this approach, for example, for selectively depleting dynamin-2, caveolin-1 and clathrin (heavy chains) [180].

5. Conclusions

The current state of knowledge of the mode of internalization of endocytic receptors still allows to individuate clathrin-mediated internalization as the most commonly used mechanism of uptake for receptor-bound materials; this mechanism appears to be predominant for receptors such as transferrin, EGFR (and possibly other receptor tyrosine kinases) and several lectins, and it can be adopted also when ligands such as cell adhesion molecules and cell penetrating peptides are used. As a general rule, clathrin-dependent internalization leads to an increasing acidic endosomal environment and a final localization of the cargo molecule/nanocarrier in lysosomes. In a logical translation of this information in terms of molecular design, if a nanocarrier surface is designed to target specific cells/locations through the use of ligands that are taken up through a clathrin-mediated mechanism, its bulk should also be capable of undergoing an acidification-induced endosomal escape.

It appears clear, however, that this picture is more complicated: not only do several ligands (cell penetrating peptides, cell adhesion molecules) at least partially reuse non-clathrin-dependent mechanisms, and some (e.g., GPI-APs) do so predominantly, but also the nature of the internalization machinery appears increasingly redundant, with the possibility for some mechanisms to be compensated by others on inhibition.

6. Expert opinion

It is now rather widely accepted that an effective targeting of receptors because of their pathological overexpression is not sufficient to ensure the efficacy of the targeted delivery action. As the intracellular fate (e.g., possibility of exposure to acidic or aggressive environments, of exocytosis, of re-presentation on the cell membrane) of materials taken up is often linked to their mechanism of internalization, and this may be determined by the same ligand–receptor binding events used for targeting nanocarriers, the molecular design of the carrier should respond to the conditions imposed by the choice of its targeting ligands.

The knowledge of endocytic processes has advanced tremendously in the last two decades: a rather simplistic, dualistic view of endocytosis as an either receptor-based or non-receptor-based process has been replaced by a more

Table 2. Most common inhibitors used in the study of endocytic pathways [176].

Inhibitor	Inhibited mechanism	Dose/pre-incubation time	Ref.
Chlorpromazine	**Clathrin-mediated endocytosis**	65 μg/ml (30′ at 37°C)	[197]
Hypertonic challenge		(0.45 M sucrose) (30′ at 37°C)	[198]
Intracellular K^+ depletion		K^+ free buffer (30′ at 37°C)	[199]
Filipin Nystatin	**Caveolin-mediated endocytosis** by binding to 3β-hydroxysterols (distinction between clathrin and *caveolae*)	5 μg/ml (30′ at 37°C)	[193,198,200]
Methyl-β-cyclodextrin	Cholesterol depletion	10 mM (10 – 30′ at 37°C)	[201]
Genestein	Tyrosine-kinase inhibitor disrupting actin network at the site of endocytosis and inhibiting dynamin II	400 μmol/l (120′ at 37°C)	
Dynasore	Membrane-permeable inhibitor of dyamin I and dynamin II	80 μM (10′ at 37°C)	[202]
Clostridium difficile toxin	Inhibition of Rho proteins, surely involved in IL2Rβ-mediated uptake, but possibly used in most clathrin-independent mechanisms	0.74 nM (60′ at 37°C)	[203]
Okadaic acid	Stimulated trafficking and internalization of *caveolae*	1 μM (30′ at 37°C)	[201,204]
Dimethylamiloride	**Macropinocytosis** by inhibiting the Na+/H+ exchange protein in the plasma membrane → cytosolic acidification. LY294002, wortmannin and 3-methyladenine inhibit phosphoinositide 3-kinase (PI 3-kinase); 3-methyladenine inhibits fusion of endosomes into a macropinosome, while the other two inhibit also the formation of endosomes. Amilorides inhibit protein kinase-C	100 μM (60′ at 37°C)	[205]
Ethylisopropylamiloride		100 μM (60′ at 37°C)	[206]
LY294002		20 μM (60′ at 37°C)	[207]
Wortmannin		10 nM (60′ at 37°C)	[208]
3-Methyladenine		10 mM (15′ at 37°C)	[209]
Low temperature (4°C)	**All energy dependent processes**	4°C	[210]
Sodium azide		100 mM (30′ at 37°C)	[210]
Chlororquine	**Acidification of endosomes** (endolysosomal pathway affected in its late stages). They highlight phenomena of acidification-induced endosomal escape and may inhibit receptor recycling	200 μM (30′ at 37°C)	[211]
Bafilomycin A1		200 nM (30′ at 37°C)	[98]
Brefeldin A		100 nM (30′ at 37°C)	[212]
Monensin		5 μM	[164]
Nigericin		5 μM	[213]
Cytochalasin *D (actin filament barbed-end capping)*	Membrane ruffling. Particularly important in macropinocytosis, ancillary in clathrin-mediated endocytosis	1 μM (30′ at 37°C)	
Latrunculin B *(actin dimer sequestering and severing)*		1 μM (30′ at 37°C)	[174]
Swinholide A *(actin monomer sequestering)*		10 nM (60′ at 37°C)	
Nocodazole	Disruption of **microtubules**	0.1 μg/ ml (30′ at 37°C) or	[24,183,214]
		1.5 μg/ ml (60′)	[34]
Colchicine		1 μM (120′ at 37°C)	[215-217]

realistic ensemble of a variety of machineries. Despite this advance, an unequivocal classification of the most commonly targeted receptors has been only partially achieved. The issues to solve are complex: for example, cell type specificities may cause the same receptor to adopt different internalization pathways in different cells, therefore the choice of a tumoral model cell line does not ensure the possibility of extending the results to other cells. Also, the interconnection between different endocytic mechanisms and their redundancy add further variables to the overall picture; it is foreseeable that a systems approach will be very helpful to rationalize it.

In terms of future developments for targeted drug delivery, it appears quite clear that the use of peptidic targeting groups (e.g., CAMs and CPPs) is an effective approach and has great potential, for example for the non-viral delivery of nucleic acids or of transcription factors, but does still require considerable effort to understand the complex web of signaling that regulates the endosomal evolution. At present, most evidence points to the contemporaneous presence of different endocytic processes, which may (clathrin) or may not (e.g., caveolin) be followed by acidification. The possibility of designing ligand/carrier combinations orientated to one specific uptake mechanism would be key for improving the design of non-viral carriers.

More also has to be done to understand the environmental conditions typical of non-acidifying endosomes (e.g., caveosomes), in order to develop responsive nanocarriers capable of endosomal escape under non-acidic conditions.

Declaration of interest

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