

# Expert Opinion

1. Introduction
2. Gateways to cellular access
3. Internalization receptors used in targeted drug delivery
4. The endocytic detective's tools
5. Conclusions
6. Expert opinion

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

Noha M Zaki & Nicola Tirelli<sup>†</sup>

<sup>†</sup>University of Manchester, School of Materials and School of Biomedicine, Stopford Building, Laboratory of Polymers and Biomaterials, Oxford Road, Manchester M13 9PT, UK

**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

*Expert Opin. Drug Deliv.* (2010) 7(8):895-913

### 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,

**informa**  
healthcare

**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 endocytosed 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.

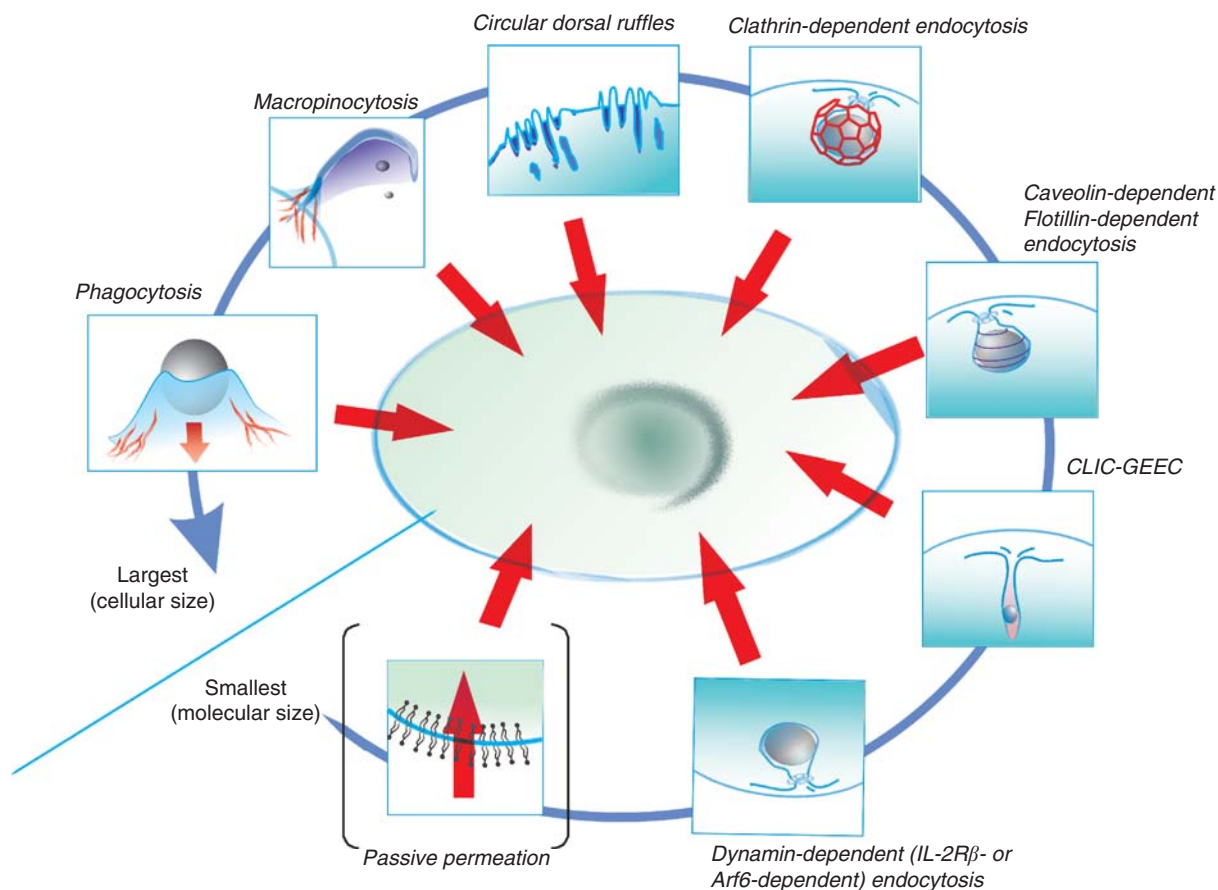
### 2.1 Phagocytosis

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  $\mu\text{m}$ ), 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  $\beta$ 2-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  $\beta$ 2-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].

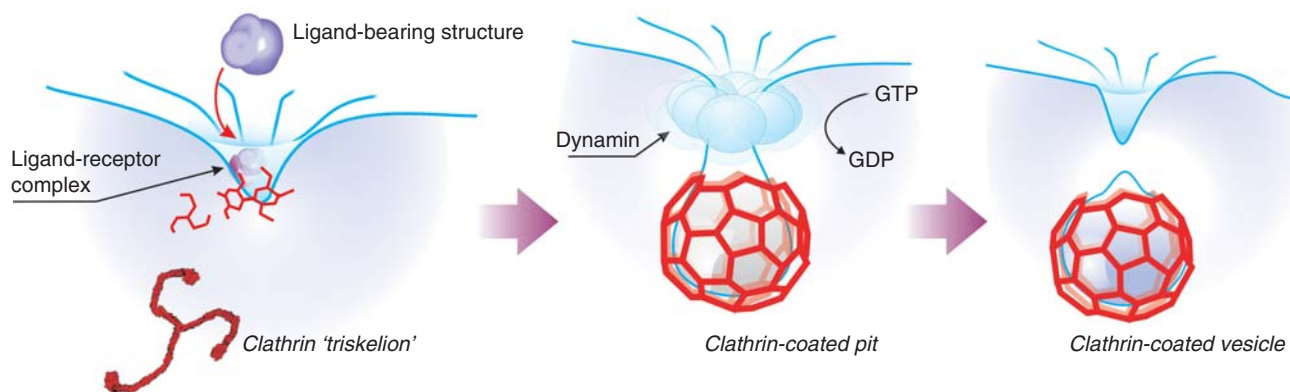
The internalization through caveolin-mediated endocytosis opens the way to the possibility of avoiding the aggressive lysosomal compartment and also, if caveosomes are involved, any form of acidification; this suggests that the cell penetration of several pathogens and/or toxins, such as SV40 viruses [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<sub>9</sub>) 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: flotillin-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 clathrin-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-fueled 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  $\beta$  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  $\beta$  (IL-2R $\beta$ ) [55], whose internalization was recognized to be dependent on the presence of lipid rafts before an *ad hoc* mechanism was postulated [56], and the  $\gamma$ c cytokine receptor [57], which is part of the same family of cytokine receptors. The high-affinity receptor Fc $\epsilon$ RI, 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),  $\beta_1$  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 melantoferrin). These non-heme glycoproteins transport iron ( $\text{Fe}^{3+}$ ) 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 endocytic 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 endocytosed material to the brain parenchyma is the main mechanism of iron transport to the

brain [72]. During the endosomal permanence holotransferrin dissociates into  $\text{Fe}^{\text{III}}$ , which is reduced to  $\text{Fe}^{\text{II}}$  and rapidly exocytosed, 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  $\beta$ -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 (multi-ligand 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 EGFR (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 down-regulation, 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 EGFR) 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 EGFR 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 down-regulation 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 clathrin-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 ( $\rightarrow$ galactosamine) [100,101], or *Dolichos biflorus* agglutinin ( $\rightarrow$ *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<sub>2</sub>) 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<sub>12</sub>) 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<sub>12</sub>-modified 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, endocytosed 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  $\alpha$ , $\beta$ -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  $\beta_1$ -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 dependence)

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 (*caBmpr1a*) 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  $\alpha$ -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<sub>9</sub>) 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<sub>9</sub> 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- $\alpha$  and FR- $\beta$ , 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- $\alpha$  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- $\alpha$  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- $\beta$ , 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, Esmaili *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 enhanced 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 $\lambda_{\max}$ (nm)	Emission $\lambda_{\max}$ (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]
Cholera 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	<b>Clathrin-mediated endocytosis</b>	65 µg/ml (30' at 37°C)	[197]
Hypertonic challenge		(0.45 M sucrose) (30' at 37°C)	[198]
Intracellular K <sup>+</sup> depletion		K <sup>+</sup> free buffer (30' at 37°C)	[199]
Filipin	<b>Caveolin-mediated endocytosis</b> by binding to 3β-hydroxysterols (distinction between clathrin and <i>caveolae</i> )	5 µg/ml (30' at 37°C)	[193,198,200]
Nystatin			
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 <i>caveolae</i>	1 µM (30' at 37°C)	[201,204]
Dimethylamiloride	<b>Macropinocytosis</b> by inhibiting the Na <sup>+</sup> /H <sup>+</sup> 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)	<b>All energy dependent processes</b>	4°C	[210]
Sodium azide		100 mM (30' at 37°C)	[210]
Chloroquine	<b>Acidification of endosomes</b> (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 ( <i>actin filament barbed-end capping</i> )	Membrane ruffling. Particularly important in macropinocytosis, ancillary in clathrin-mediated endocytosis	1 µM (30' at 37°C)	
Latrunculin B ( <i>actin dimer sequestering and severing</i> )		1 µM (30' at 37°C)	[174]
Swinholide A ( <i>actin monomer sequestering</i> )		10 nM (60' at 37°C)	
Nocodazole	Disruption of <b>microtubules</b>	0.1 µg/ ml (30' at 37°C) or	[24,183,214]
Colchicine		1.5 µg/ ml (60')	[34]
		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

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

### Bibliography

1. Labhasetwar V. Nanotechnology for drug and gene therapy: the importance of understanding molecular mechanisms of delivery. *Curr Opin Biotech* 2005;16(6):674-80
2. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003;422(6927):37-44
3. Jovic M, Sharma M, Rahajeng J, Caplan S. The early endosome: a busy sorting station for proteins at the crossroads. *Histol Histopath* 2010;25(1):99-12
4. Surti N, Naik S, Bagchi T, et al. Intracellular delivery of nanoparticles with biological systems. *Biomaterials* 2008;9(1):217-23
5. Basarkar A, Misra A. Intracellular delivery of nanoparticles of an antiasthmatic drug. *AAPS PharmSciTech* 2008;9(1):217-23
6. Deretic V, Singh S, Master S, et al. Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol* 2006;8(5):719-27
7. Huynh KK, Grinstein S. Regulation of vacuolar pH and its modulation by some microbial species. *Microbiol Mol Biol Rev* 2007;71(3):452-62
8. Cho YW, Kim JD, Park K. Polycation gene delivery systems: escape from endosomes to cytosol. *J Pharm Pharmacol* 2003;55(6):721-34
9. Engqvist-Goldstein AEY, Drubin DG. Actin assembly and endocytosis: from yeast to mammals. *Annu Rev Cell Dev Biol* 2003;19:287-332
10. Robertson AS, Smythe E, Ayscough KR. Functions of actin in endocytosis. *CMLS-Cell. Mol Life Sci* 2009;66(13):2049-65
11. Tsujita K, Suetsugu S, Sasaki N, et al. Coordination between the actin cytoskeleton and membrane deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. *J Cell Biol* 2006;172(2):269-79
12. Orth JD, Mcniven MA. Dynamin at the actin-membrane interface. *Curr Opin Cell Biol* 2003;15(1):31-9
13. Cao H, Orth JD, Chen J, et al. Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. *Mol Cell Biol* 2003;23(6):2162-70
14. Mcniven MA, Thompson HM. Vesicle formation at the plasma membrane and trans-Golgi network: the same but different. *Science* 2006;313(5793):1591-4
15. Hansen CG, Nichols BJ. Molecular mechanisms of clathrin-independent endocytosis. *J Cell Sci* 2009;122(11):1713-21
16. Gleich GJ, Adolphson CR. The eosinophilic leukocyte - structure and function. *Adv Immunol* 1986;39:177-253
17. Everts V, Vanderzee E, Creemers L, Beertsen W. Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling. *Histochem J* 1996;28(4):229-45
18. Smith ME. Phagocytosis of myelin in demyelinating disease: a review. *Neurochem Res* 1999;24(2):261-8
19. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem* 2009;78:857-902
20. Kerr MC, Teasdale RD. Defining macropinocytosis. *Traffic* 2009;10(4):364-71
21. Shao YF, Akmentin W, Toledo-Aral JJ, et al. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. *J Cell Biol* 2002;157(4):679-91
22. Meier O, Boucke K, Hammer SV, et al. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol* 2002;158(6):1119-31
23. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 2004;10(3):310-15
24. Goncalves C, Mennesson E, Fuchs R, et al. Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. *Mol Ther* 2004;10(2):373-85
25. Orth JD, Krueger EW, Weller SG, Mcniven MA. A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. *Cancer Res* 2006;66(7):3603-10
26. Orth JD, Mcniven MA. Get off my back! Rapid receptor internalization through circular dorsal ruffles. *Cancer Res* 2006;66(23):11094-6
27. Goldstein JL, Brown MS, Anderson RGW, et al. Receptor-mediated endocytosis - concepts emerging from the LDL receptor system. *Annu Rev Cell Biol* 1985;1:1-39
28. Motley A, Bright NA, Seaman MNJ, Robinson MS. Clathrin-mediated endocytosis in AP-2-depleted cells. *J Cell Biol* 2003;162(5):909-18
29. Vieira AV, Lamaze C, Schmid SL. Control of EGF receptor signaling by

- clathrin-mediated endocytosis. *Science* 1996;274(5295):2086-9
30. Laporte SA, Oakley RH, Zhang J, et al. The beta(2)-adrenergic receptor/beta arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* 1999;96(7):3712-17
  31. Maudsley S, Pierce KL, Zamah AM, et al. The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem* 2000;275(13):9572-80
  32. Kirchhausen T. Imaging endocytic clathrin structures in living cells. *Trends Cell Biol* 2009;19(11):596-605
  33. Sorkin A, Von Zastrow M. Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol* 2002;3(8):600-14
  34. Parton RG, Simons K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol* 2007;8(3):185-94
  35. Scherer PE, Lewis RY, Volonte D, et al. Cell-type and tissue-specific expression of caveolin-2 – caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J Biol Chem* 1997;272(46):29337-46
  36. Lajoie P, Goetz JG, Dennis JW, Nabi IR. Lattices, rafts, and scaffolds: domain regulation of receptor signaling at the plasma membrane. *J Cell Biol* 2009;185(3):381-5
  37. Landth T. From entangled membranes to eclectic morphologies – cubic membranes as subcellular space organizers. *FEBS Lett* 1995;369(1):13-7
  38. Oh P, McIntosh DP, Schnitzer JE. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J Cell Biol* 1998;141(1):101-14
  39. Pelkmans L, Burli T, Zerial M, Helenius A. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell* 2004;118(6):767-80
  40. Pelkmans L, Kartenbeck J, Helenius A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* 2001;3(5):473-83
  41. Sandvig K, Torgersen ML, Raa HA, Van Deurs B. Clathrin-independent endocytosis: from nonexistent to an extreme degree of complexity. *Histochem Cell Biol* 2008;129(3):267-76
  42. Norkin LC, Anderson HA, Wolfrom SA, Oppenheim A. Caveolar endocytosis of Simian virus 40 is followed by brefeldin A-sensitive transport to the endoplasmic reticulum, where the virus disassembles. *J Virol* 2002;76(10):5156-66
  43. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol* 1998;141(4):905-15
  44. Sottile J, Chandler J. Fibronectin matrix turnover occurs through a caveolin-1-dependent process. *Mol Biol Cell* 2005;16(2):757-68
  45. Sharma DK, Brown JC, Cheng ZJ, et al. The glycosphingolipid, lactosylceramide, regulates beta(1)-integrin clustering and endocytosis. *Cancer Res* 2005;65(18):8233-41
  46. Frick M, Bright NA, Riento K, et al. Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. *Curr Biol* 2007;17(13):1151-6
  47. Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* 2006;8(1):46-54
  48. Ait-Slimane T, Galmes R, Trugnan G, Maurice M. Basolateral internalization of GPI-anchored proteins occurs via a clathrin-independent flotillin-dependent pathway in polarized hepatic cells. *Mol Biol Cell* 2009;20(17):3792-800
  49. Lu YJ, Low PS. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv Drug Deliv Rev* 2002;54(5):675-93
  50. Cheng ZJ, Singh RD, Sharma DK, et al. Distinct mechanisms of clathrin-independent endocytosis have unique sphingolipid requirements. *Mol Biol Cell* 2006;17(7):3197-10
  51. Sabharanjak S, Sharma P, Parton RG, Mayor S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* 2002;2(4):411-23
  52. Kirkham M, Fujita A, Chadda R, et al. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. *J Cell Biol* 2005;168(3):465-76
  53. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* 2007;8(8):603-12
  54. Lundmark R, Doherty GJ, Howes MT, et al. The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. *Curr Biol* 2008;18(22):1802-8
  55. Grassart A, Dujecourt A, Lazarow PB, et al. Clathrin-independent endocytosis used by the IL-2 receptor is regulated by Rac1, Pak1 and Pak2. *EMBO Rep* 2008;9(4):356-62
  56. Matko J, Bodnar A, Vereb G, et al. GPI-microdomains (membrane rafts) and signaling of the multi-chain interleukin-2 receptor in human lymphoma/leukemia T cell lines. *Eur J Biochem* 2002;269(4):1199-208
  57. Sauvonnnet N, Dujecourt A, Dautry-Varsat A. Cortactin and dynamin are required for the clathrin-dependent endocytosis of gamma c cytokine receptor. *J Cell Biol* 2005;168(1):155-63
  58. Fattakhova G, Masilamani M, Borrego F, et al. The high-affinity immunoglobulin-E receptor (Fc epsilon RI) is endocytosed by an AP-2/clathrin-independent, dynamin-dependent mechanism. *Traffic* 2006;7(6):673-85
  59. Peng M, Yin N, Zhang W. Endocytosis of Fc alpha R is clathrin and dynamin dependent, but its cytoplasmic domain is not required. *Cell Res* 2010;20(2):223-37
  60. Naslavsky N, Weigert R, Donaldson JG. Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol Biol Cell* 2004;15(8):3542-52
  61. Houndolo T, Boulay PL, Claing A. G protein-coupled receptor endocytosis in ADP-ribosylation factor 6-depleted cells. *J Biol Chem* 2005;280(7):5598-604
  62. Kang YS, Zhao XH, Lovaas J, et al. Clathrin-independent internalization of normal cellular prion protein in neuroblastoma cells is associated with the Arf6 pathway. *J Cell Sci* 2009;122(22):4062-9
  63. Lau AW, Chou MM. The adaptor complex AP-2 regulates post-endocytic trafficking through the non-clathrin Arf6-dependent endocytic pathway. *J Cell Sci* 2008;121(24):4008-17

64. Qian ZM, Li HY, Sun HZ, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol Rev* 2002;54(4):561-87
65. Daniels TR, Delgado T, Helguera G, Penichet ML. The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. *Clin Immunol* 2006;121(2):159-76
66. O'DONNELL KA, Yu DN, Zeller KI, et al. Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis. *Mol Cell Biol* 2006;26(6):2373-86
67. Bartlett DW, Davis ME. Physicochemical and biological characterization of targeted, nucleic acid-containing nanoparticles. *Bioconjug Chem* 2007;18(2):456-68
68. Pan X, Guan J, Yoo J-W, et al. Cationic lipid-coated magnetic nanoparticles associated with transferrin for gene delivery. *Int J Pharm* 2008;358(1-2):263-70
69. Mishra V, Mahor S, Rawat A, et al. Targeted brain delivery of AZT via transferrin anchored pegylated albumin nanoparticles. *J Drug Target* 2006;14(1):45-53
70. Roberts RL, Fine RE, Sandra A. Receptor-mediated endocytosis of transferrin at the blood-brain-barrier. *J Cell Sci* 1993;104:521-32
71. Visser CC, Voorwinden LH, Crommelin DJA, et al. Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm Res* 2004;21(5):761-9
72. Fishman JB, Rubin JB, Handrahan JV, et al. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. *J Neurosci Res* 1987;18(2):299-304
73. Morgan EH, Moos T. Mechanism and developmental changes in iron transport across the blood-brain barrier. *Dev Neurosci* 2002;24(2-3):106-13
74. Broadwell RD, Bakercairns BJ, Friden PM, et al. Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Exp Neurol* 1996;142(1):47-65
75. Friden PM, Walus LR, Musso GF, et al. Antitransferrin receptor antibody and antibody-drug conjugates cross the blood-brain-barrier. *Proc Natl Acad Sci USA* 1991;88(11):4771-5
76. Huwyler J, Wu DF, Partridge WM. Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci USA* 1996;93(24):14164-9
77. Skarlatos S, Yoshikawa T, Partridge WM. Transport of [I-125] transferrin through the rat blood-brain-barrier. *Brain Res* 1995;683(2):164-71
78. Sahoo SK, Labhasetwar V. Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. *Mol Pharm* 2005;2(5):373-83
79. Xu L, Pirollo KF, Tang WH, et al. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. *Hum Gene Ther* 1999;10(18):2941-52
80. Kompella UB, Sundaram S, Raghava S, Escobar ER. Luteinizing hormone-releasing hormone agonist and transferrin functionalizations enhance nanoparticle delivery in a novel bovine ex vivo eye model. *Mol Vis* 2006;12(134-135):1185-98
81. Roepstorff K, Grovdal L, Grandal M, et al. Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer. *Histochem Cell Biol* 2008;129(5):563-78
82. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Mat Biotechnol* 2005;23(9):1147-57
83. Lurje G, Lenz HJ. EGFR signaling and drug discovery. *Oncology* 2009;77(6):400-10
84. Song S, Liu D, Peng J, et al. Peptide ligand-mediated liposome distribution and targeting to EGFR expressing tumor in vivo. *Int J Pharm* 2008;363(1-2):155-61
85. Tseng C-L, Wang T-W, Dong G-C, et al. Development of gelatin nanoparticles with biotinylated EGF conjugation for lung cancer targeting. *Biomaterials* 2007;28(27):3996-4005
86. Saul JM, Annapragada AV, Bellamkonda RV. A dual-ligand approach for enhancing targeting selectivity of therapeutic nanocarriers. *J Control Release* 2006;114(3):277-87
87. Tseng C-L, Su W-Y, Yen K-C, et al. The use of biotinylated-EGF-modified gelatin nanoparticle carrier to enhance cisplatin accumulation in cancerous lungs via inhalation. *Biomaterials* 2009;30(20):3476-85
88. Acharya S, Dilnawaz F, Sahoo SK. Targeted epidermal growth factor receptor nanoparticle bioconjugates for breast cancer therapy. *Biomaterials* 2009;30(29):5737-50
89. Sigismund S, Argenzio E, Tosoni D, et al. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell* 2008;15(2):209-19
90. East L, Isacke CM. The mannose receptor family. *Biochim Biophys Acta* 2002;1572(2-3):364-86
91. Beltzer JP, Spiess M. In vitro binding of the asialoglycoprotein receptor to the beta adaptin of plasma-membrane coated vesicles. *EMBO J* 1991;10(12):3735-42
92. Weigel PH, Yik JH. Glycans as endocytosis signals: the cases of the asialoglycoprotein and hyaluronan/chondroitin sulfate receptors. *Biochim Biophys Acta* 2002;1572(2-3):341-63
93. Poupon V, Girard M, Legendre-Guillemin V, et al. Clathrin light chains function in mannose phosphate receptor trafficking via regulation of actin assembly. *Proc Natl Acad Sci USA* 2008;105(1):168-73
94. Irache JM, Salman HH, Gamazo C, Espuelas S. Mannose-targeted systems for the delivery of therapeutics. *Expert Opin Drug Deliv* 2008;5(6):703-24
95. Weis WI, Drickamer K. Trimeric structure of a C-type mannose-binding protein. *Structure* 1994;2(12):1227-40
96. Connaris H, Crocker PR, Taylor GL. Enhancing the receptor affinity of the sialic acid-binding domain of vibrio cholerae sialidase through multivalency. *J Biol Chem* 2009;284(11):7339-51
97. Srinivas O, Larriue P, Duverger E, et al. Synthesis of glycocluster-tumor antigenic peptide conjugates for dendritic cell targeting. *Bioconjug Chem* 2007;18(5):1547-54
98. Issa MM, Koping-Hoggard M, Tommeras K, et al. Targeted gene delivery with trisaccharide-substituted

- chitosan oligomers in vitro and after lung administration in vivo. *J Control Release* 2006;115(1):103-12
99. Gabor F, Bogner E, Weissenboeck A, Wirth M. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. *Adv Drug Deliv Rev* 2004;56(4):459-80
100. Petrossian K, Banner LR, Oppenheimer SB. Lectin binding and effects in culture on human cancer and non-cancer cell lines: examination of issues of interest in drug design strategies. *Acta Histochem* 2007;109(6):491-500
101. Sakuma S, Yano T, Masaoka Y, et al. In vitro/in vivo biorecognition of lectin-immobilized fluorescent nanospheres for human colorectal cancer cells. *J Control Release* 2009;134(1):2-10
102. Gao XL, Wang T, Wu BX, et al. Quantum dots for tracking cellular transport of lectin-functionalized nanoparticles. *Biochem Biophys Res Commun* 2008;377(1):35-40
103. Russell-Jones GJ, Veitch H, Arthur L. Lectin-mediated transport of nanoparticles across Caco-2 and OK cells. *Int J Pharm* 1999;190(2):165-74
104. Yin Y, Chen D, Qiao M, et al. Preparation and evaluation of lectin-conjugated PLGA nanoparticles for oral delivery of thymopentin. *J Control Release* 2006;116(3):337-45
105. Gao X, Tao W, Lu W, et al. Lectin-conjugated PEG-PLA nanoparticles: preparation and brain delivery after intranasal administration. *Biomaterials* 2006;27(18):3482-90
106. Huang SN, Swaan PW. Involvement of a receptor-mediated component in cellular translocation of riboflavin. *J Pharmacol Exp Ther* 2000;294(1):117-25
107. Rao PN, Levine E, Myers MO, et al. Elevation of serum riboflavin carrier protein in breast cancer. *Cancer Epidemiol Biomark* 1999;8(11):985-90
108. Holladay SR, Yang ZF, Kennedy MD, et al. Riboflavin-mediated delivery of a macromolecule into cultured human cells. *Biochim Biophys Acta* 1999;1426(1):195-204
109. Maurer ME, Cooper JA. Endocytosis of megalin by visceral endoderm cells requires the Dab2 adaptor protein. *J Cell Sci* 2005;118(22):5345-55
110. Zhai XY, Nielsen R, Birn H, et al. Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney. *Kidney Int* 2000;58(4):1523-33
111. Russell-Jones GJ, Arthur L, Walker H. Vitamin B12-mediated transport of nanoparticles across Caco-2 cells. *Int J Pharm* 1999;179(2):247-55
112. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. *Mol Biol Cell* 2004;15(1):176-88
113. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nat Cell Biol* 2006;8(11):1223-34
114. Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. *Traffic* 2006;7(1):14-21
115. Altankov G, Grinnell F. Fibronectin receptor internalization and AP-2 complex reorganization in potassium-depleted fibroblasts. *Exp Cell Res* 1995;216(2):299-309
116. De Deyne PG, O'NEILL A, Resneck WG, et al. The vitronectin receptor associates with clathrin-coated membrane domains via the cytoplasmic domain of its beta(5) subunit. *J Cell Sci* 1998;111:2729-40
117. Liu L, He B, Liu WM, et al. Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *J Biol Chem* 2007;282(43):31631-42
118. Upla P, Marjomaki V, Kankaanpaa P, et al. Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. *Mol Biol Cell* 2004;15(2):625-36
119. Caswell P, Norman J. Endocytic transport of integrins during cell migration and invasion. *Trends Cell Biol* 2008;18(6):257-63
120. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003;3(5):362-74
121. Rathinam R, Alahari SK. Important role of integrins in the cancer biology. *Cancer Metastasis Rev* 2010;29(1):223-37
122. Lutolf MP, Raeber GP, Zisch AH, et al. Cell-responsive synthetic hydrogels. *Adv Mater* 2003;15(11):883-92
123. Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. *J Biomed Mater Res* 2002;60(2):217-23
124. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol* 1997;15(6):542-6
125. Schifferers RM, Koning GA, Ten Hagen TLM, et al. Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin. *J Control Release* 2003;91(1-2):115-22
126. Bibby DC, Talmadge JE, Dalal MK, et al. Pharmacokinetics and biodistribution of RGD-targeted doxorubicin-loaded nanoparticles in tumor-bearing mice. *Int J Pharm* 2005;293(1-2):281-90
127. Liu PF, Wang HZ, Li YG, Duan YR. Preparation of DHAQ-loaded PLA-PLL-RGD nanoparticles and comparison of antitumor efficacy to hepatoma and breast carcinoma. *J Macromol Sci A* 2009;46(10):1024-9
128. Danhier F, Vroman B, Lecouturier N, et al. Targeting of tumor endothelium by RGD-grafted PLGA-nanoparticles loaded with Paclitaxel. *J Control Release* 2009;140(2):166-73
129. Wender PA, Galliher WC, Goun EA, et al. The design of guanidinium-rich transporters and their internalization mechanisms. *Adv Drug Deliv Rev* 2008;60(4-5):452-72
130. Frankel AD, Pabo CO. Cellular uptake of the TAT protein from human immunodeficiency virus. *Cell* 1988;55(6):1189-93
131. Futaki S, Suzuki T, Ohashi W, et al. Arginine-rich peptides – an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* 2001;276(8):5836-40
132. Wender PA, Mitchell DJ, Pattabiraman K, et al. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci USA* 2000;97(24):13003-8
133. Futaki S, Nakase I, Tachikoro A, et al. Arginine-rich peptides and their



- internalization mechanisms. *Biochem Soc Trans* 2007;35:784-7
134. Derossi D, Chassaing G, Prochiantz A. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol* 1998;8(2):84-7
  135. Thoren PEG, Persson D, Karlsson M, Norden B. The antennapedia peptide penetratin translocates across lipid bilayers – the first direct observation. *FEBS Lett* 2000;482(3):265-8
  136. Derossi D, Joliet AH, Chassaing G, Prochiantz A. The 3rd helix of the antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 1994;269(14):10444-50
  137. Suzuki T, Futaki S, Niwa M, et al. Possible existence of common internalization mechanisms among arginine-rich peptides. *J Biol Chem* 2002;277(4):2437-43
  138. Torchilin VP. Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. *Adv Drug Deliv Rev* 2008;60(4-5):548-58
  139. Richard JP, Melikov K, Vives E, et al. Cell-penetrating peptides – a reevaluation of the mechanism of cellular uptake. *J Biol Chem* 2003;278(1):585-90
  140. Duchardt F, Fotin-Mleczek M, Schwarz H, et al. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* 2007;8(7):848-66
  141. Khalil IA, Kogure K, Futaki S, Harashima H. High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. *J Biol Chem* 2006;281(6):3544-51
  142. Khalil IA, Kogure K, Futaki S, et al. Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery. *Gene Ther* 2007;14(8):682-9
  143. Tkachenko AG, Xie H, Liu Y, et al. Cellular trajectories of peptide-modified gold particle complexes: comparison of nuclear localization signals and peptide transduction domains. *Bioconjug Chem* 2004;15(3):482-90
  144. Zelphati O, Szoka FC Jr. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharm Res* 1996;13(9):1367-72
  145. Park YJ, Liang JF, Ko KS, et al. Low molecular weight protamine as an efficient and nontoxic gene carrier: in vitro study. *J Gene Med* 2003;5(8):700-11
  146. Zorko M, Langel U. Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv Drug Deliv Rev* 2005;57(4):529-45
  147. Yandek LE, Pokorny A, Floren A, et al. Mechanism of the cell-penetrating peptide transportan 10 permeation of lipid bilayers. *Biophys J* 2007;92(7):2434-44
  148. Watkins CL, Brennan P, Fegan C, et al. Cellular uptake, distribution and cytotoxicity of the hydrophobic cell penetrating peptide sequence PFVYLI linked to the proapoptotic domain peptide PAD. *J Control Release* 2009;140(3):237-44
  149. Saalik P, Padari K, Niinep A, et al. Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways. *Bioconjug Chem* 2009;20(5):877-87
  150. Salazar MD, Ratnam M. The folate receptor: what does it promise in tissue-targeted therapeutics? *Cancer Metastasis Rev* 2007;26(1):141-52
  151. Nakashima-Matsushita N, Homma T, Yu S, et al. Selective expression of folate receptor beta and its possible role in methotrexate transport in synovial macrophages from patients with rheumatoid arthritis. *Arthritis Rheum* 1999;42(8):1609-16
  152. Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. *Adv Drug Deliv Rev* 2004;56(8):1067-84
  153. Leamon CP, Reddy JA. Folate-targeted chemotherapy. *Adv Drug Deliv Rev* 2004;56(8):1127-41
  154. Low PS, Henne WA, Doornweerd DD. Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases. *Acc Chem Res* 2008;41(1):120-9
  155. Zhao XB, Li H, Lee RJ. Targeted drug delivery via folate receptors. *Expert Opin Drug Deliv* 2008;5(3):309-19
  156. Schneider RL, Schmitt F, Frochot C, et al. Design, synthesis, and biological evaluation of folic acid targeted tetraphenylporphyrin as novel photosensitizers for selective photodynamic therapy. *Bioorg Med Chem* 2005;13(8):2799-808
  157. Zheng Y, Cai Z, Song X, et al. Receptor mediated gene delivery by folate conjugated N-trimethyl chitosan in vitro. *Int J Pharm* 2009;382(1-2):262-9
  158. Esmaeili F, Dinarvand R, Ghahremani MH, et al. Cellular cytotoxicity and in-vivo biodistribution of docetaxel poly(lactide-co-glycolide) nanoparticles. *AntiCancer Drug* 2010;21(1):43-52
  159. Pan J, Feng S-S. Targeted delivery of paclitaxel using folate-decorated poly (lactide)-vitamin E TPGS nanoparticles. *Biomaterials* 2008;29(17):2663-72
  160. Wang Y, Yu L, Han L, et al. Difunctional pluronic copolymer micelles for paclitaxel delivery: synergistic effect of folate-mediated targeting and pluronic-mediated overcoming multidrug resistance in tumor cell lines. *Int J Pharm* 2007;337(1-2):63-73
  161. Mcneeley KM, Karathanasis E, Annapragada AV, Bellamkonda RV. Masking and triggered unmasking of targeting ligands on nanocarriers to improve drug delivery to brain tumors. *Biomaterials* 2009;30(23-24):3986-95
  162. Quintana A, Raczka E, Piehler L, et al. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. *Pharm Res* 2002;19(9):1310-16
  163. Kukowska-Latalo JF, Candido KA, Cao Z, et al. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Res* 2005;65(12):5317-24
  164. Veldhoen S, Laufer SD, Trampe A, Restle T. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. *Nucleic Acids Res* 2006;34(22):6561-73
  165. Park JS, Han TH, Lee KY, et al. N-acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: endocytosis, exocytosis and drug release. *J Control Release* 2006;115(1):37-45
  166. Coester C, Nayyar P, Samuel J. In vitro uptake of gelatin nanoparticles by murine dendritic cells and their intracellular

- localisation. *Eur J Pharm Biopharm* 2006;62(3):306-14
167. Richard JP, Melikov K, Brooks H, et al. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* 2005;280(15):15300-6
168. Perumal OP, Inapagolla R, Kannan S, Kannan RM. The effect of surface functionality on cellular trafficking of dendrimers. *Biomaterials* 2008;29(24-25):3469-76
169. Huth US, Schubert R, Peschka-Suss R. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. *J Control Release* 2006;110(3):490-504
170. Win KY, Feng SS. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials* 2005;26(15):2713-22
171. Hu Y, Xie J, Tong YW, Wang CH. Effect of PEG conformation and particle size on the cellular uptake efficiency of nanoparticles with the HepG2 cells. *J Control Release* 2007;118(1):7-17
172. Huang M, Ma Z, Khor E, Lim LY. Uptake of FITC-chitosan nanoparticles by A549 cells. *Pharm Res* 2002;19(10):1488-94
173. Chavanpatil MD, Khair A, Panyam J. Surfactant-polymer nanoparticles: a novel platform for sustained and enhanced cellular delivery of water-soluble molecules. *Pharm Res* 2007;24(4):803-10
174. Qaddoumi MG, Ueda H, Yang J, et al. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. *Pharm Res* 2004;21(4):641-8
175. Davda J, Labhasetwar V. Characterization of nanoparticle uptake by endothelial cells. *Int J Pharm* 2002;233(1-2):51-9
176. Vercauteren D, Vandenbroucke RE, Jones AT, et al. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol Ther* 2010;18(3):561-9
177. Munn AL, Silveira L, Elgort M, Payne GS. Viability of clathrin heavy-chain-deficient *Saccharomyces Cerevisiae* is compromised by mutations at numerous loci – implications for the suppression hypothesis. *Mol Cell Biol* 1991;11(8):3868-78
178. Nelson KK, Lemmon SK. Suppressors of clathrin deficiency – overexpression of ubiquitin rescues lethal strains of clathrin-deficient *Saccharomyces Cerevisiae*. *Mol Cell Biol* 1993;13(1):521-32
179. Huang FT, Khvorova A, Marshall W, Sorkin A. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* 2004;279(16):16657-61
180. Spoden G, Freitag K, Husmann M, et al. Clathrin- and caveolin-independent entry of human papillomavirus type 16-involvement of tetraspanin-enriched microdomains (TEMs). *PLoS One* 2008;3(10):e3313
181. Panyam J, Labhasetwar V. Dynamics of endocytosis and exocytosis of poly(D, L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. *Pharm Res* 2003;20(2):212-20
182. Huth US, Schubert R, Peschka-Suss R. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. *J Control Release* 2006;110(3):490-504
183. Van Der MA, Huth US, Hafele SY, et al. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. *Pharm Res* 2007;24(8):1590-8
184. Nayak S, Lee H, Chmielewski J, Lyon LA. Folate-mediated cell targeting and cytotoxicity using thermoresponsive microgels. *J Am Chem Soc* 2004;126(33):10258-9
185. De Diesbach P, N'KULI F, Berens C, et al. Receptor-mediated endocytosis of phosphodiester oligonucleotides in the HepG2 cell line: evidence for non-conventional intracellular trafficking. *Nucleic Acids Res* 2002;30(7):1512-21
186. Des Rieux A, Ragnarsson EG, Gullberg E, et al. Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium. *Eur J Pharm Sci* 2005;25(4-5):455-65
187. Kim SH, Choi HJ, Lee KW, et al. Interaction of SPIN90 with syndapin is implicated in clathrin-mediated endocytic pathway in fibroblasts. *Genes Cells* 2006;11(10):1197-11
188. Kahn E, Menetrier F, Vejux A, et al. Flow cytometry and spectral imaging multiphoton microscopy analysis of CD36 expression with quantum dots 605 of untreated and 7-ketocholesterol-treated human monocytic cells. *Anal Quant Cytol Histol* 2006;28(6):316-30
189. Mo Y, Lim LY. Preparation and in vitro anticancer activity of wheat germ agglutinin (WGA)-conjugated PLGA nanoparticles loaded with paclitaxel and isopropyl myristate. *J Control Release* 2005;107(1):30-42
190. Yumoto R, Nishikawa H, Okamoto M, et al. Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN. *Am J Physiol Lung C* 2006;290(5):L946-955
191. Amidi M, Romeijn SG, Borchard G, et al. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. *J Control Release* 2006;111(1-2):107-16
192. Cherukuri A, Frye J, French T, et al. FITC-poly-D-lysine conjugates as fluorescent probes to quantify hapten-specific macrophage receptor binding and uptake kinetics. *Cytometry* 1998;31(2):110-24
193. Rejman J, Braganzzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther* 2005;12(3):468-74
194. Huang M, Khor E, Lim LY. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. *Pharm Res* 2004;21(2):344-53
195. Ma Z, Lim LY. Uptake of chitosan and associated insulin in Caco-2 cell monolayers: a comparison between chitosan molecules and chitosan nanoparticles. *Pharm Res* 2003;20(11):1812-19
196. Serpe L, Guido M, Canaparo R, et al. Intracellular accumulation and cytotoxicity of doxorubicin with different pharmaceutical formulations in human cancer cell lines. *J Nanosci Nanotechnol* 2006;6(9-10):3062-9
197. Gotte M, Sofeu Feugaing DD, Kresse H. Biglycan is internalized via a chlorpromazine-sensitive route. *Cell Mol Biol Lett* 2004;9(3):475-81
198. Qaddoumi MG, Gukasyan HJ, Davda J, et al. Clathrin and caveolin-1 expression in primary pigmented rabbit

- conjunctival epithelial cells: role in PLGA nanoparticle endocytosis. *Mol. Vis* 2003;9(68-69):559-68
199. Bauer IW, Li SP, Han YC, et al. Internalization of hydroxyapatite nanoparticles in liver cancer cells. *J Mater Sci Mater Med* 2008;19(3):1091-5
200. Manunta M, Nichols BJ, Tan PH, et al. Gene delivery by dendrimers operates via different pathways in different cells, but is enhanced by the presence of caveolin. *J Immunol Methods* 2006;314(1-2):134-46
201. Thomsen P, Roepstorff K, Stahlhut M, Van Deurs B. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol Biol Cell* 2002;13(1):238-50
202. Macia E, Ehrlich M, Massol R, et al. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell* 2006;10(6):839-50
203. Van Hamme E, Dewerchin HL, Cornelissen E, et al. Clathrin- and caveolae-independent entry of feline infectious peritonitis virus in monocytes depends on dynamin. *J Gen Virol* 2008;89:2147-56
204. Parton RG, Joggerst B, Simons K. Regulated internalization of caveolae. *J Cell Biol* 1994;127(5):1199-15
205. Shepherd PR, Reaves BJ, Davidson HW. Phosphoinositide 3-kinases and membrane traffic. *Trends Cell Biol* 1996;6(3):92-7
206. Nakase I, Niwa M, Takeuchi T, et al. Cellular uptake of arginine-rich peptides: Roles for macropinocytosis and actin rearrangement. *Mol Ther* 2004;10(6):1011-22
207. Corvera S, Czech MP. Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction. *Trends Cell Biol* 1998;8(11):442-6
208. Ui M, Okada T, Hazeki K, Hazeki O. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* 1995;20(8):303-7
209. Araki N, Hamasaki M, Egami Y, Hatae T. Effect of 3-methyladenine on the fusion process of macropinosomes in EGF-stimulated A431 cells. *Cell Struct Funct* 2006;31(2):145-57
210. Enriquez De A, Diebold Y, Calonge M, et al. Chitosan nanoparticles as a potential drug delivery system for the ocular surface: toxicity, uptake mechanism and in vivo tolerance. *Invest Ophthalmol Vis Sci* 2006;47(4):1416-25
211. Dijkstra J, Van Galen M, Scherphof GL. Effects of ammonium chloride and chloroquine on endocytic uptake of liposomes by Kupffer cells in vitro. *Biochim Biophys Acta* 1984;804(1):58-67
212. Hunziker W, Whitney JA, Mellman I. Brefeldin A and the endocytic pathway possible implications for membrane traffic and sorting. *FEBS Lett* 1992;307(1):93-6
213. Oishi M, Kataoka K, Nagasaki Y. pH-responsive three-layered PEGylated polyplex micelle based on a lactosylated ABC triblock copolymer as a targetable and endosome-disruptive nonviral gene vector. *Bioconjug Chem* 2006;17(3):677-88
214. Hashimoto M, Morimoto M, Saimoto H, et al. Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjug Chem* 2006;17(2):309-16
215. Dijkstra J, Van Galen M, Scherphof G. Effects of (dihydro)cytochalasin B, colchicine, monensin and trifluoperazine on uptake and processing of liposomes by Kupffer cells in culture. *Biochim Biophys Acta* 1985;845(1):34-42
216. Ramge P, Unger RE, Oltrogge JB, et al. Polysorbate-80 coating enhances uptake of polybutylcyanoacrylate (PBCA)-nanoparticles by human and bovine primary brain capillary endothelial cells. *Eur J Neurosci* 2000;12(6):1931-40
217. Boyd AE III, Bolton WE, Brinkley BR. Microtubules and beta cell function: effect of colchicine on microtubules and insulin secretion in vitro by mouse beta cells. *J Cell Biol* 1982;92(2):425-34

#### Affiliation

Noha M Zaki<sup>1</sup> & Nicola Tirelli<sup>†2</sup>

<sup>†</sup>Author for correspondence

<sup>1</sup>Ain Shams University, Department of Pharmaceutics, Faculty of Pharmacy, Monazamet El Wehda El Afrikia St, El Abbassia, Cairo, Egypt  
<sup>2</sup>University of Manchester, School of Materials and School of Biomedicine, Laboratory of Polymers and Biomaterials, Stopford Building, Oxford Road, Manchester M13 9PT, UK  
 E-mail: Nicola.tirelli@manchester.ac.uk