Uptake and Intracellular Trafficking of Nanocarriers

Helene Andersen, Ladan Parhamifar and S. Moein Moghimi

Abstract Nanocarriers are widely used for delivery of therapeutic and modulatory agents to eukaryotic cells and specific intracellular compartments. Nanocarrier internalization proceeds via different routes and predominantly via clathrin-coated pits, lipid rafts/caveolae endocytosis and macropinocytosis/phagocytosis, depending on the cell type as well as the physicochemical properties of the nanocarrier. The intracellular fate of the nanocarrier is not only dependent on the mode of entry, but may also be modulated by prior surface modification of nanocarriers with organelle-specific localization ligands. This chapter discusses important methodological aspects for studying cellular uptake and intracellular trafficking of nanocarriers.

Keywords Nanocarriers · Endocytosis · Intracellular trafficking

Abbreviation

CME	Clathrin-mediated endocytosis
ER	Endoplasmic reticulum
GFP	Green fluorescence protein
MOC	Manders overlap coefficient
PALM	Photoactivated localization microscopy
PCC	Pearson co-localization coefficient
PEI	Polyethylenimine
STORM	Stochastic optical reconstruction microscopy
TEM	Transmission electron microscopy

H. Andersen · L. Parhamifar · S. Moein Moghimi (✉)
Nanomedicine Research Group, Centre for Pharmaceutical Nanotechnology and Nanotoxicology, Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
e-mail: moien.moghimi@sund.ku.dk

A. Prokop et al. (eds.), *Intracellular Delivery II*,
Fundamental Biomedical Technologies 7, DOI: 10.1007/978-94-017-8896-0_6,

1 Introduction

There have been numerous attempts to develop nanocarriers, which can not only improve drug solubilisation and delivery to different eukarocytic, but also target the desired intracellular compartments (Treuel et al. 2013). Several cellular barriers, however, need to be crossed before nanoparticles can reach their designated intracellular targets. Accordingly, a carrier may be designed to only enter specific cells in relevant tissues and to enter different cells through a specific endocytic pathway (Wang 2012). Likewise, intracellular transport mechanism of various nanocarriers have also received considerable attention (Treuel et al. 2013; Sakhrani and Padh 2013).

Various methods have been developed to study the mechanisms by which nanocarriers are internalized by cells of different origin and to follow their intracellular trafficking (Vercauteren et al. 2012). Here we describe the most studied endocytic pathways and discuss some of the major barriers for uptake and trafficking of nanocarriers. Advantages and disadvantages of commonly used methods for nanocarrier trafficking studies are also discussed.

2 Endocytosis

For nanocarriers to deliver and release their cargo at an intracellular target site the carriers need to enter the cell by crossing the plasma membrane. Most nanoparticles are believed to be internalized by endocytosis (Canton et al. 2012). Endocytosis is an energy-dependent process where particles are internalized in small vesicles. The mostly studied endocytic pathways are clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis, but more pathways have been identified that includes clathrin- and caveolae-independent endocytosis and phagocytosis (Doherty and McMahon 2009) (Fig. 1).

2.1 Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis (CME) is initiated at clathrin-coated pits in plasma membrane (Maxfield et al. 2004). Once the vesicle is formed in a dynamin-dependent manner (Hinshaw 2000), it is uncoated and followed by fusion with or maturation into early or recycling endosomes (Lemmon 2001; Ma et al. 2002; Maxfield et al. 2004). Clathrin is important for the initiation of invaginations at the membrane level and for the formation of the endocytic vesicles. Other assembly proteins are also involved in the formation of invaginations (Brodsky et al. 2001; Kirchhausen 1999).

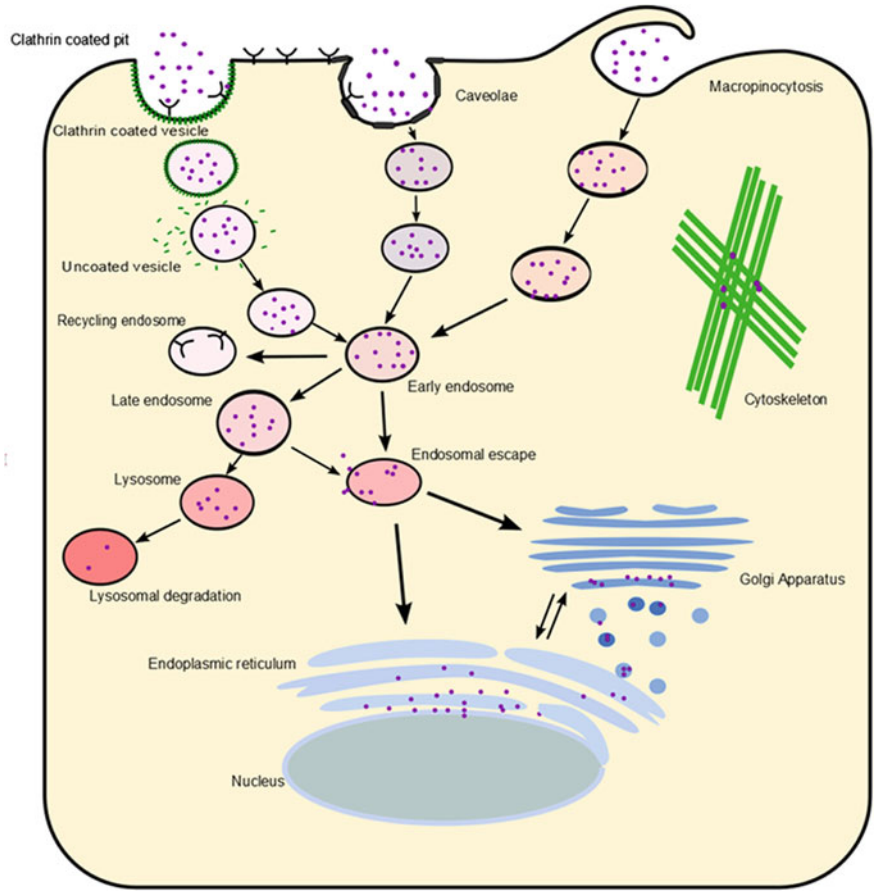

Fig. 1 Schematic overview of intracellular trafficking pathways

The clathrin coated vesicles have been demonstrated to be of various sizes ranging from 50 to 300 nm. Particles reported to be internalized by CME are generally up to 200 nm in diameter (Ehrlich et al. 2004). However, some studies have indicated that larger particles may also be taken up by CME (Moreno-Ruiz et al. 2009; Veiga et al. 2005). CME, however, is important when it comes to cellular uptake and sorting of nutrients, plasma membrane proteins and lipids (Conner et al. 2003).

2.2 Caveolae-Mediated Endocytosis

Caveolae-mediated endocytosis initiates from cholesterol-rich areas on the plasma membrane (Khalil et al. 2006a). Formation of the invaginations is dynamin- and

actin-dependent and the vesicles formed are reported to be in 60–80 nm size range (Canton et al. 2012; Hinshaw 2000; Parton et al. 2007). Theses vesicles are coated primarily by caveolin-1, which is responsible for the structure of the endocytic vesicle, and caveolin-2. However, the role of the latter is still not clear (Canton et al. 2012). The endocytic vesicles have been reported to be transported either to the caveosome or to the early endosomes (Parton et al. 2003; Pelkmans et al. 2001). Nanocarriers internalized by caveolae-mediated endocytosis are reported to be transported to endoplasmic reticulum and Golgi apparatus and some nanocarriers are also detected in the nucleus (Harris et al. 2002; Luetterforst et al. 1999; Pelkmans et al. 2001). For gene delivery vectors, caveolae-mediated endocytosis is the preferred route of internalization for efficient delivery and transcription of the exogenous DNA (Gabrielson et al. 2009; van der Aa et al. 2007) as this may substantially avoid lysosomal routing and subsequent lysosomal degradation of the nucleic acids (Harris et al. 2002).

2.3 Macropinocytosis

Macropinocytosis is an actin-dependent membrane ruffling, which results in the formation of large endocytic vesicles known as macropinosomes (Kerr et al. 2009). Macropinocytosis plays an important role in cellular uptake of fluids (Conner et al. 2003). The reported size of the macropinosomes varies, but have been demonstrated to be up to several micrometres, which is much larger than vesicles from other endocytic pathways (Jones 2007). The size of the formed vesicles further allows an opportunity for internalization of larger particles that cannot enter via clathrin- and caveolae-mediated endocytosis. Indeed, some pathogens use macropinocytosis to facilitate entry to different cells (Mercer et al. 2009).

2.4 Factors Influencing the Endocytic Pathway

Several factors have been reported to influence nanoparticle endocytosis. These include nanoparticle physicochemical properties such as size, shape, surface charge and ligand coating (Canton et al. 2012; Zhao et al. 2011).

A study by Rejman et al. (2004) demonstrated that while uptake of particles up to 200 nm was dependent on microtubule, their corresponding larger particles (500 nm) were not. This study further indicated that smaller particles where mainly internalized by CME, whereas the larger particles entered cells through cholesterol-dependent endocytosis and to a lesser extent were routed to the endo-lysosomal pathway (Rejman et al. 2004). However, the literature is not consistent in correlating nanoparticle size with internalization pathway. For instance, some reports have indicated that particles up to 500 nm can be internalized by caveolae-

mediated endocytosis (Georgieva et al. 2011; Rejman et al. 2004), while others report that only particles up to 100 nm can be taken up by this route (Wang et al. 2009). These discrepancies may be due to different experimental setups, nanoparticle surface properties and the cell types examined.
Although the size of the nanoparticles can influence the endocytic pathway, many particles are not uniform in size and have a broad size distribution, which might render them to enter cells by different endocytic pathways, thus complicating the analysis of internalization routes. Others have reported that the shape of the nanoparticles determines the endocytic pathway for internalization (Gratton et al. 2008).

The uptake of particulate carriers of various sizes is also dependent on the cell type (Massignani et al. 2009; Nakai et al. 2003). Zauner et al. (2001) presented a study where they investigated the cellular uptake of microsphere of various sizes by different cell lines. Not all the cell lines were capable of internalizing particles above 1 μm, while all the tested cell lines could take up particles of 20 nm (Zauner et al. 2001). Furthermore, the net surface charge of the particles has also been suggested to influence the internalization pathway. Charged polystyrene or gold particles are reported to internalize to a higher degree than electrically neutral particles (Thorek et al. 2008; Villanueva et al. 2009). Also positively charged particles have been reported to be internalized easier than their corresponding anionic counterparts (Chen et al. 2011). This has been suggested to be due to ionic interaction between the positively charged particle and the negatively charged plasma membrane. In addition to the extent of the particle uptake, the surface charge of the particles may further regulate the pathway of internalisation (Harush-Frenkel et al. 2008; Zuidam et al. 2000). It has been demonstrated that dendrimers coupled to different functional groups enter the cell by different pathways dependent on the functional group. Dendrimers with amine or hydroxyl functional groups enter cells through both CME and caveolae-independent pathways, while dendrimers with functional carboxyl group mainly enter the cells via caveolae-mediated endocytosis (Perumal et al. 2008).

Georgieva et al. (2011) reported that 500 nm nanoparticles coated with either PEI or prion proteins were internalized through different endocytic pathways compared with uncoated particles. The coating of the particles also had an effect on the extent of particles that were co-localizing with recycling endosomes (Georgieva et al. 2011). The percentage of particles removed from cells by exocytosis may also dependent on the size of the particles. For example, the fraction of small 14 nm gold nanoparticles exocytosed was higher than gold particles with a diameter of 100 nm. Even though the percentage of particles exocytosed was reported to be cell-type dependent, the trend is the same and a higher fraction of the small particles being exocytosed (Chithrani et al. 2007). Not only the coating, but also the density of the molecules used for coating can play an important role in determining the endocytic pathway. Coating of liposomes with high density of the cell penetrating peptide R8, shifted the endocytic pathway towards macropinocytosis compared with liposomes coated with low density of the peptide, which were taken up by CME (Khalil et al. 2006b). A study by Li et al. (2011) indicated

that PEI/DNA polyplexes are internalized by both clathrin- and caveolae-mediated endocytosis. However, when PEI was chemically modified with the natural polymer cyclodextran, the uptake mechanism was shifted toward caveolae-mediated endocytosis in HEK293T cells, thus demonstrating the role of charge and composition of the carrier in modulating the internalization route (Li et al. 2011).

3 Methods for Identifying Endocytic Pathways

Two methods are commonly used to study the pathways of nanocarriers internalization by cells. These include exclusion methodologies as well as co-localization studies, where overlap between fluorescently labelled particles and fluorescently labelled proteins are investigated by qualitative fluorescent microscopy.

3.1 Exclusion Methodologies

For exclusion determination, the nanocarrier should be fluorescently labelled so the uptake can be measured by flow cytometry or quantitative microscopy (Vercauteren et al. 2012). Several methods to exclude endocytic pathways have been demonstrated. For instance, siRNA can be used to decrease the expression of proteins required for the specific endocytic pathway in question (Zaki and Tirelli 2010). The use of siRNA may induce less cytotoxicity and be more specific than alternative methods used for the exclusion assays (Spoden et al. 2008). Caveolin-1 and dynamin-2 are among the successful proteins that have been down-regulated with siRNA (Huang et al. 2004). However, knockdown of proteins required for certain endocytic pathways by siRNA technology, may lead to up-regulation of some of the other endocytic pathways to compensate (Damke et al. 1995).

The most commonly used form of exclusion assays entail chemically inhibiting specific endocytic pathways. Table 1 lists some of the most commonly used inhibitors. The endocytosis inhibitors do not show exclusive specificity and many of the inhibitors of CME have been reported to cause reorganization of the actin skeleton (Ivanov 2008). Changes in the cytoskeleton might also affect other endocytic pathways, which are reported to be actin-dependent. Especially hypertonic sucrose as inhibitor for CME has been reported to be non-specific (Ivanov 2008). Also one of the mostly used inhibitors for caveolae-mediated endocytosis, methyl-β-cyclodextrin, which causes acute depletion of cholesterol, has been reported to significantly affect the cytoskeleton and also to inhibit CME and macropinocytosis (Kanzaki et al. 2002; Rodal et al. 1999). Inhibitors of macropinocytosis are also non-specific (Ivanov 2008). Even though, it is difficult to inhibit one endocytic pathway without affecting other pathways, the inhibitors are widely useful when studying internalization of nanocarriers.

Table 1 Chemical inhibitors commonly used for exclusion assays

Inhibitor	Pathway	Mechanism	References
Chlorpromazin	CME	Inhibit clathrin coated pit formation	Wang et al. (1993)
Potassium depletion	CME	Translocation of clathrin from plasma membrane to cytosol	Hansen et al. (1993)
Weak acids	CME	Inhibiting the budding of clathrin coated pits	Sandvig et al. (1987)
Nystatin	Raft/caveolae	Flattens the caveolae	Ros-Baro et al. (2001)
Lovastatin	Raft/caveolae	Inhibits HMG-CoA reductase	Liao and Laufs (2005)
Simvastatin	Raft/caveolae	Inhibits HMG-CoA reductase	Liao and Laufs (2005)
Pravastatin	Raft/caveolae	Inhibits HMG-CoA reductase	Liao and Laufs (2005)
Filipin	Raft/caveolae	Changes the structure of caveolae	Ros-Baro et al. (2001)
Okadaic acid	Raft/cacaolae	Removes caveolae from cell surface	Parton et al. (1994)
Amiloride and amiloride analogs	Macropinocytosis and phagocytosis (CME)	Inhibit sodium-proton exchange	Nakase et al. (2004), West et al. (1989)
Genistein	Caveolae	Inhibits tyrosine kinase and thereby phosphorylation of caveolin	Akiyama et al. (1987), Parton et al. (1994)
Nocodazol	Macropinocytosis, phagocytosis	Destabilize micotubuli	Mettlen et al. (2006)
Taxol	Macropinocytosis, phagocytosis	Microtubule stabilizing	de et al. (1981), Roberts et al. (1982)
Latrunculin	Macropinocytosis, phagocytosis—maybe unselective inhibitor	Blocks F-actin polymerization by binding to monomeric F-actin	Mettlen et al. (2006)
Cytochalasin	Macropinocytosis, phagocytosis—maybe unselective inhibitor	Blocks F-action polymerization by binding to the end of fast growing actin filament	Brett et al. (1984)
Wortmanin	Macropinocytosis and phagocytosis	Inhibits PI3K	Mettlen et al. (2006), Salh et al. (1998)
LY294002	Macropinocytosis and phagocytosis	Inhibits PI3K	(Salh et al. 1998)
Methyl- β -cyclodextrin	Raft/caveolae	Removes cholesterol from plasma membrane, flatten and induce mislocalization of caveolin	Kilsdonk et al. (1995), Westermann et al. (2005)

(continued)

Table 1 (continued)

Inhibitor	Pathway	Mechanism	References
Phenylarsine oxide	CME	Reduces energy stores inhibits protein synthesis	Bradley et al. (1993), Visser et al. (2004), Yumoto et al. (2006)
Cholesterol oxidase	Raft/caveolae	Removes caveolin from plasma membrane	Smart et al. (1994)
Hypertonic sucrose	CME	Prevents internalization of receptor bound peptide by inhibiting endosome formation	Daukas et al. (1985)
NH_4Cl	CME	Inhibits budding of clathrin coated pits	Sandvig et al. (1987)
Dansylcadaverine	Phagocytosis	Inhibits formation of phosohatidylcholine	Bradley et al. (1993), Garcia et al. (1982)
Rimantadine	Phagocytosis	Inhibits formation of phosohatidylcholine	Garcia et al. (1982)
Putrescine			Bradley et al. (1993)
N-ethylamaleimide	Caveolae/transcytosis		Schnitzer et al. (1995)

Some of the chemical inhibitors have been reported to induce cell type-dependent cytotoxicity. This can result in incorrect interpretation. Accordingly, appropriate concentration of inhibitors must be selected to optimize the conditions prior to testing (Vercauteren et al. 2010). When analyzing exclusion assays by flow cytometry, positive controls such as transferrin and folic acid, which are known to be internalized by clathrin- and caveolae-mediated endocytosis, respectively, should be included (Rothberg et al. 1990; Schmid 1997). However, flow cytometry may not necessarily distinguish between cells with internalized particles and those with surface bound particles (Ogris et al. 2000; Salvati et al. 2011).

In addition, genetically-modified cell lines have been used to exclude endocytic pathways (Ilina et al. 2012; Rejman et al. 2004). For example, Ilina et al. (2012) reported particle uptake studies with genetically-modified cells where dynamin-dependent and clathrin-mediated endocytic pathways were not operative. They further reported that the use of chemical inhibitors and the genetic blockage do not result in the same level of decrease in transfection efficiency (Ilina et al. 2012). These results further indicate that the interpretation of the inhibition assays can be difficult, and several methods should be considered before reaching a conclusion about the uptake mechanism of particles in specific cell lines. One of the problems with inhibition of endocytic pathways by mutant cell lines is the possibility of another pathway becoming increasingly active, which may aid the uptake of nanocarriers (Damke et al. 1995).

When identifying the endocytic pathway important for efficient delivery of pDNA by PEI, many contradicting reports have been published. Von Gersdorff et al. (2006) reported that clathrin-mediated endocytosis is required for efficient transfection, whereas Hufnagel et al. (2009) reported that fluid phase uptake plays an important role for efficient transfection with PEI/DNA polyplexes. However, it has also been demonstrated that blocking of caveolae-mediated endocytosis can inhibit expression of exogenous DNA (Gabrielson et al. 2009).

Uptake of fluorescently-labeled nanoparticles is usually measured by flow cytometry at various time points after addition of particles. Not all fluorophores are suitable for use in investigating cellular uptake through endocytosis. An example is fluorescein which has been reported to show decreased brightness when pH is below 9 and almost fully quenched at pH 6, which could result in inaccurate interpretation of the results when the particles have reached the acidic environment of endosomes and lysosomes (Geisow 1984).

3.2 Co-localization Microscopy Studies for Uptake Mechanism

Co-localization studies with fluorescence microscopy depend on the availability of fluorescently labeled nanocarriers and fluorescently labeled markers for the specific endocytic pathways that are being investigated (Vercauteren et al. 2012).

A commonly used method is to add the nanocarrier to living cells, which is followed by fixation at various time points after addition. After fixation, immunostaining of the specific endocytic marker is performed to detect possible co-localization between nanocarrier and the marker for the endocytic pathway. Several fixative agents have been reported to cause artifacts, which need to be considered when analyzing the data (Lundberg et al. 2001; Pearson 2007). To avoid such artifacts live-cell imaging may be applied.

To study co-localization by live-cell imaging, the marker protein has to be tagged with an appropriate fluorescent marker. This may include stably transfected cell lines where the marker proteins are coupled to a fluorescent tag such as the Green Fluorescence Protein (GFP) or the Red Fluorescent Protein. However, it is important to control the expression of the fluorescently-labeled marker proteins, since their overexpression might increase the activity of an endocytic pathway, which does not have the same activity in the parental cell line. Furthermore, co-localization between two molecules acquisition, pre-processing of the image and also sample preparation are very important steps to overcome possible artifacts (Abraham et al. 2010; Bolte et al. 2006).

3.3 Other Methods to Study Internalization

Other methods have also been used to analyze the internalization of particles in the nanometer range. Scanning electron microscopy, transmission electron microscopy and atomic force microscopy have been applied to detect reorganization of the plasma membrane (Georgieva et al. 2011; Leroueil et al. 2007). The re-organization of the membrane when endocytosis is initiated varies between the different pathways and the electron density at the invagination sites can be distinguished by electron microscopy thus making it possible to distinguish between endocytic pathways (Doherty and McMahon 2009). Dynamic surface enhanced Raman spectroscopy has also been used to investigate nanoparticle (e.g., gold nanoparticles and carbon nanotubes) internalization and intracellular transport pathways (Ando et al. 2011).

4 Intracellular Transport

From the point when the endocytic vesicles are detached from the membrane the carrier has to be transported to the targeted cellular compartment. Several mechanisms of how the cargo is transported from early endosomes through late endosomes to lysosomes or recycling endosomes have been suggested (Gruenberg et al. 2004; Luzio et al. 2007; Saftig et al. 2009). Both the possibility of gradual maturation and fusions of compartments have been suggested (Stoorvogel et al. 1991). It has further been demonstrated that endosomes and lysosomes can communicate

via small vesicles in cell free systems, which indicate that it is not only gradual maturation that takes place (Vida et al. 1999).

Nanoparticles have been reported to accumulate in the peri-nuclear region shortly after internalization (Bae et al. 2012; Bregar et al. 2013; Fichter et al. 2013; Kim et al. 2012; Suh et al. 2003). The movement of carriers has in several cases been demonstrated to be dependent on polymerization of either actin or microtubule (Suh et al. 2003). To investigate the role of microtubule or actin in the cytosolic transport of nanocarriers chemical inhibitors of polymerization of tubulin or actin have been reported to interrupt the transport. PEI/DNA polyplexes have been reported to be dependent on actin during cellular uptake, whilst the intracellular trafficking is suggested to be microtubule dependent. In addition, a lactosylated derivate of PEI was reported to be transported along microtubule (Grosse et al. 2007). Similarly, liposomes have been reported to be transported along microtubule during cellular transport. However, it has been demonstrated that the transfection efficiency is enhanced both when disrupting or stabilizing microtubule, because liposomes no longer are transported to the lysosomes. R8-coated liposomes only reached the periphery of the nuclear when microtubule was not disrupted by nocodazole (Hasegawa et al. 2001; Khalil et al. 2008). On the other hand, PEI-mediated transfection was almost eliminated when disrupting microtubule with nocodazole treatment in COS-7 cells (Grosse et al. 2007). Also free plasmid DNA has been reported to be dependent of microtubule-mediated transport on the route to the nucleus (Vaughan and Dean 2006). Suh et al. (2003) reported that PEI/DNA polyplexes were transported along microtubule. The transport was directed towards the nucleus and carried out by motor protein along microtubule (Suh et al. 2003). However, it should be considered that when destabilizing the microtubule and actin network the cell morphology is highly affected. This can lead to changes in both cell uptake and intracellular transport of nanoparticles (Dos et al. 2011).

Caveolae-mediated endocytosis has in many cases been reported to be an endocytic pathway where delivery to endo-lysosomal pathway can be avoided and trafficking occurs from the caveosome to endoplasmic reticulum and Golgi apparatus (Badizadegan et al. 2000; Lencer et al. 1999). Currently, this transport mechanism has not been completely elucidated. Particles can also be transported between ER and Golgi apparatus. This event has been demonstrated to take advantage of the normal cellular trafficking between the two organelles. Glycofect particles has been demonstrated to be transported in COP I coated vesicle from Golgi to ER (Fichter et al. 2013).

Liposomes internalized by macropinocytosis have also been reported to avoid lysosomal degradation and to be present in the cytosol after endocytosis. When the liposomes where internalized by macropinocytosis the transfection efficiency was significantly higher than when internalized through CME (Khalil et al. 2006b).

4.1 Endosomal Escape

To avoid degradation in the acidic lysosome environment nanoparticles have to escape from early endosomes. One suggested mechanism is the proton-sponge hypothesis and applicable to cationic species. This hypothesis was first suggested in 1997 by Behr and colleagues. Here, polycationic species were suggested to induce influx of negatively charged ions (e.g., chlorides) into the endosomes. This influx is followed by water due to change in osmotic pressure. This may result in endosome swelling and eventual rupture, resulting in cytosolic release of the polycation (Behr 1997). A recent work, however, reported that the lysosomal pH does not change after treating cells with PEI, thus suggesting that the proton sponge hypothesis may be an inadequate explanation for endolysosomal release (Benjaminsen et al. 2013).

Polycations have previously been described to be able to cause perturbations of lipid bilayers, which may be an alternative mechanism by which nanocarriers may escape endo-lysosomes (Bieber et al. 2002). Addition of cationic lipids to lipid carriers has resulted in endosomal escape probably due to destabilization of the lipid bilayer of endosomes (Wasungu et al. 2006). Akita et al. (2010) reported that a pH-sensitive fusogenic peptide could be modified for endosomal escape. One example is the GALA peptide, which can destabilize the lipid bilayer resulting in successful endosomal escape (Akita et al. 2010).

4.2 Cytosolic Transport

After release from the endosomes nanoparticles must reach their designated intracellular targets. For example, if pDNA is delivered the release should happen in the periphery of the nucleus so the DNA is not degraded before it reaches the nucleoplasm (Lechardeur et al. 2005). For cytoplasmic dissociation biodegradable carriers have been designed. The biodegradable carriers will be gradually degraded in the cytoplasm resulting in release of the cargo (Gosselin et al. 2001).

To reach the cellular compartment where the cargo is going to be released an increasing amount of research has been put into targeting the organelle of interest (Sakhrani and Padh 2013). For instance, the ER retention signal consisting of four amino acids (KDEL) has been used to target ER. By targeting ER the endo-lysosomal pathway might be bypassed and lysosomal degradation can be avoided (Acharya et al. 2013; Wang et al. 2013). Mitochondria play an important role in initiation of apoptosis, regulating calcium homeostasis, removal of reactive oxygen species and in ATP synthesis. Hence, targeting of mitochondria through mitochondrial targeting signal peptide can be very important in treatment of various conditions (Heller et al. 2012; Sakhrani and Padh 2013).

4.3 Nuclear Entry

If the cargo of the nanocarrier requires transport to the nucleus, passing the nuclear envelope is a major barrier for efficient delivery. On the nuclear envelope, nuclear pore complexes are widely distributed (Grossman et al. 2012). Molecules that enter the nucleus have to pass through the channel of this complex. Small molecules up to 9 nm in diameter can freely diffuse through the pore channel (Paine et al. 1975), whereas molecules up to 39 nm are transported through the complex by an energy-dependent transport (Pante et al. 2002).

DNA needs to be transported through the nuclear pore complex, but the mechanism behind this still needs further investigation. Some reports suggest that only during mitosis DNA can enter the nucleus or that the transfection efficiency is dependent on the cell cycle, but microinjection of DNA in the cytoplasm has shown that the exogenous DNA can be transcribed in non-dividing cells (Brunner et al. 2000; Dean et al. 2005; Pollard et al. 1998).

It has been reported that a nuclear localization sequence added to the nanocarriers can increase the transport to the nucleus. Also a DNA sequence coding for a binding site for NFkB can increase the transport of DNA to the nucleus resulting in an increase in transfection (Breuzard et al. 2008; Dean et al. 2005).

Entry to the nucleus has been under intense investigation and many contradicting reports have been published. For example, PEI has been suggested to be transported to the nucleus, only to be present in the nucleus after mitosis, whereas other studies have indicated the inability of PEI to reach the nucleus (Brunner et al. 2000; Dowty et al. 1995; Larsen et al. 2012). Not many studies have evaluated the mechanism by which a nanocarrier enters the nucleus, but it has been suggested that polycations might rupture the membrane to access the nucleoplasm (Godbey et al. 1999). The size of the nanocarriers can also be a major obstacle in the transport through the NPC, since only molecules with a diameter of less than 40 nm can be transported through the nuclear pore complex and most nanocarriers have been reported to be above 100 nm (Pante et al. 2002).

5 Techniques to Investigate Cellular Localization of Nanocarriers

Investigations of intracellular trafficking of nanocarriers are in many cases carried out by fluorescence microscopy. However, it is important to interpret the microscopy data with care, since artifacts may be generated and the interpretation of microscopy images can be very subjective (Bolte et al. 2006).

Fixation of cells before staining is widely used, but as previously mentioned the fixation can induce artifacts. Live-cell imaging decreases the amount of artifact that usually can be observed after a fixation procedure. However, to be able to detect which organelles the nanocarrier is transported to it is necessary to use cell

permanent organelle specific dyes or to label organelle specific proteins with GFP or similar species (Vercauteren et al. 2012).

There exist several types of fluorescence microscopes. When using confocal microscopes the end product is an image where only light from the focal plane is emitted. For wide-field microscopes also light from the out of focus planes is visible in the final image (Bolte et al. 2006). Images from wide-field microscopes require deconvolution of the images to eliminate the out of focus blur. Especially, if quantifying the co-localization of nanocarriers with organelle markers wide-field images need to be deconvolved but it can also be an advantage to deconvolve confocal images (Bolte et al. 2006; Scriven et al. 2008). Deconvolution requires an accurate point-spread-function, which can be determined with use of various algorithms calculating point-spread-function (PSF) in various ways. The PSF should also be determined for the optical conditions used in experiments (McNally et al. 1999). Co-localization can also be determined visually. An overlap of two channels where the detected light is represented in two different colors will result in color change if there is an overlap between the fluorophores. This, however, is a subjective way of determining overlaps between channels and is only qualitative (Bolte et al. 2006; Dunn et al. 2011). In recent years quantitative algorithms have been developed to calculate the degree of co-localization.

The degree of co-localization can be calculated according to various coefficients. The most commonly used are the Pearson co-localization coefficient (PCC) and the Manders overlap coefficient (MOC). The PCC identify co-localization when a pixel from two different has the same intensity, whereas MOC detect overlap when fluorescence in a pixel from two channels is detected (Bolte et al. 2006; Manders et al. 1992). When using PCC it is important that the same instrument settings are used every time and when imaging nanocarriers their might still be a different in intensity in both the nanocarrier and also in the protein labeled from cell to cell and especially between experiments (Dunn et al. 2011).

For live-cell imaging MOC is the most commonly used coefficient since intensity from organelle markers and the nanocarrier itself can vary between experiments (Dunn et al. 2011). It is important when using MOC that a background threshold is set to avoid false positive read-out, which is a major risk when calculating MOC to analyze the degree of overlap (Bolte et al. 2006; Dunn et al. 2011; Fletcher et al. 2010).

Suh et al. (2003) have performed real-time multiple particle tracking when reporting that intracellular transport of PEI/DNA polyplexes to the periphery of the nuclei is dependent on motor proteins along microtubule (Suh et al. 2003). The same group also reported that polyplexes were actively transported to the early and late endosomes and transport between the endosomes was also observed when performing real-time multiple particle tracking (Suh et al. 2012).

Single-particle tracking is another powerful tool to study the route by which one single nanocarrier is transported through the cell (Ruthardt and Brauchle 2010). This method requires a fast and sensitive camera and the efficient laser excitation. Single-particle tracking is following the trajectory for each separately visible particle. Co-localization of particles are defined as significant correlation between

the trajectories (Braeckmans et al. 2010). This method provides the possibility to investigate if the single particles are transported in the same direction and to the same organelles and will provide new insights as how a population of nanoparticles behaves when added to cells (Ruthardt and Brauchle 2010). Other microscopy methods have been applied to study the intracellular distribution of nanocarriers. Among them Raman microscopy and Fluorescence recovery after photo-bleaching have been used to investigate interaction between nanoparticles and mitochondria (Chernenko et al. 2009; Hemmerich et al. 2013; Phizicky et al. 2003).

5.1 High-Resolution Fluorescence Microscopy

Many of the traditional microscopes have a detection limit around 200 nm in the XY-plane and 500 nm in the Z-plane for optimal settings. This gives a problem when imaging nanocarriers with few hundred nanometers in diameter and single particles are not possible to image (Bolte et al. 2006).

An optimized hardware is necessary to perform high-resolution microscopy. Each step from the laser excitation to the camera acquisition has to be optimized compared with conventional microscopy. When performing high resolution microscopy on live cells there is a risk of inducing cell death due to high laser power. Photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are two of the most described high resolution microscopy techniques (Henriques et al. 2011). The super resolution techniques create a dataset by excitation of few fluorophores at a time and an image is the reconstructed based on the fluorescent particles. PALM and STORM are two very similar setup but they vary in the fluorophores that are used for the experiments. PALM uses genetically attached fluorophores such as GFP coupled proteins, whereas STORM take advantages of fluorescent dyes (Henriques et al. 2011).

5.2 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is also widely used for co-localization studies. Gold labeled antibodies against a specific antigen are a specific method to investigate the cellular localization of the protein of interest. By TEM it is possible to distinguish between various organelles and cellular compartment and the endocytic vesicles (Georgieva et al. 2011). However the interpretation of electron microscopy can be difficult and may generate artifacts during sample preparation. Sample preparation for electron microscopy includes embedding, slicing and staining of the sample before imaging and this is very time consuming compared to fluorescent imaging (Henriques et al. 2011). Nanocarriers can often be visualized on their own by negative staining.

5.3 Cellular Fractionation

Cellular fractionation has also been used to study the intracellular localization of nanocarriers. Centrifugation steps are carried out to separate the various organelles (He et al. 2013; Shi et al. 2013). For instance, Shi et al. (2013) labeled their polyplexes with [^{3}H]-DNA and [^{14}C]-PEI before adding the polyplexes to the cell growth medium. They separated the lysed cells into nuclear, light mitochondria, heavy mitochondria, microsomes and cytosolic fractions. Quantification of the radioactivity indicated that a large fraction of both PEI and DNA was detected in the nuclei fraction. This method, however, was not sufficient to demonstrate if polyplexes entered the nucleus intact or separately as DNA and/or PEI (Shi et al. 2013). A major disadvantage of this method is the risk of contaminating the different cellular compartments with parts from the other fractions.

6 Conclusion

Uptake and intracellular trafficking of nanocarriers is currently under intense investigation. Many factors can influence the endocytic pathways and need to be taken into consideration when designing assays and analyzing the data. Quantification of co-localization between nanocarriers and the marker protein can be determined in a very subjective manner by looking at the overlay images from two or more channels. However, several algorithms have been developed to give a more objective quantification of the co-localization. Also exclusion assays when studying the uptake of nanocarriers can be difficult to interpret with certainty, since inhibiting the selected pathways can result in unwanted cellular responses such as toxic responses or undesired increased activation of alternative pathways.

Acknowledgment Financial support by the Danish Agency for Science, Technology and Innovation (Det Frie Forskningsråd for Teknologi og Produktion) reference 274-08-0534 is gratefully acknowledged.

References

Abraham T, Allan SE, Levings MK (2010) Deconvolution and chromatic aberration corrections in quantifying colocalization of a transcription factor in three-dimensional cellular space. Micron 41(6):633–640

Acharya S, Hill RA (2013) High efficacy gold-KDEL peptide-siRNA nanoconstruct-mediated transfection in C2C12 myoblasts and myotubes. Nanomedicine doi: 10.1016/j.nano.2013.07.015.doi epub ahead of print

Ando J, Fujita K, Smith NI, Kawata S (2011) Dynamic SERS imaging of cellular transport pathways with endocytosed gold nanoparticles. Nano Lett 11:5344–5348

Akita H, Kogure K, Moriguchi R et al (2010) Nanoparticles for ex vivo siRNA delivery to dendritic cells for cancer vaccines: programmed endosomal escape and dissociation. J Control Rel 143(3):311–317
Akiyama T, Ishida J, Nakagawa S et al (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262(12):5592–5595
Badizadegan K, Wolf AA, Rodighiero C et al (2000) Floating cholera toxin into epithelial cells: functional association with caveolae-like detergent-insoluble membrane microdomains. Int J Med Microbiol 290(4–5):403–408
Bae YM, Park YI, Nam SH et al (2012) Endocytosis, intracellular transport, and exocytosis of lanthanide-doped upconverting nanoparticles in single living cells. Biomaterials 33(35):9080–9086
Behr JP (1997) The proton sponge: a trick to enter cells the viruses did not exploit. Chimia 51(1–2):34–36
Benjaminsen RV, Mattebjerg MA, Henriksen JR et al (2013) The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH. Mol Ther 21(1):149–157
Bieber T, Meissner W, Kostin S et al (2002) Intracellular route and transcriptional competence of polyethylenimine-DNA complexes. J Control Release 82(2–3):441–454
Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224(Pt 3):213–232
Bradley JR, Johnson DR, Pober JS (1993) Four different classes of inhibitors of receptor-mediated endocytosis decrease tumor necrosis factor-induced gene expression in human endothelial cells. J Immunol 150(12):5544–5555
Braeckmans K, Buyens K, Naeye B et al (2010) Advanced fluorescence microscopy methods illuminate the transfection pathway of nucleic acid nanoparticles. J Control Rel 148(1):69–74
Bregar VB, Lojk J, Sustar V et al (2013) Visualization of internalization of functionalized cobalt ferrite nanoparticles and their intracellular fate. Int J Nanomed 8:919–931
Brett JG, Godman GC (1984) Macrovacuolation induced by cytochalasin: its relation to the cytoskeleton; morphological and cytochemical observations. Tissue Cell 16(3):311–324
Breuzard G, Tertil M, Goncalves C et al (2008) Nuclear delivery of NFkappaB-assisted DNA/polymer complexes: plasmid DNA quantitation by confocal laser scanning microscopy and evidence of nuclear polyplexes by FRET imaging. Nucleic Acids Res 36(12):e71
Brodsky FM, Chen CY, Knuehl C et al (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. Annu Rev Cell Dev Biol 17:517–568
Brunner S, Sauer T, Carotta S et al (2000) Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther 7(5):401–407
Canton I, Battaglia G (2012) Endocytosis at the nanoscale. Chem Soc Rev 41(7):2718–2739
Chen L, Mccrate JM, Lee JC et al (2011) The role of surface charge on the uptake and biocompatibility of hydroxyapatite nanoparticles with osteoblast cells. Nanotechnology 22(10):105708
Chernenko T, Matthaus C, Milane L et al (2009) Label-free Raman spectral imaging of intracellular delivery and degradation of polymeric nanoparticle systems. ACS Nano 3(11):3552–3559
Chithrani BD, Chan WC (2007) Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Lett 7(6):1542–1550
Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422(6927):37–44
Damke H, Baba T, van der Bliek AM et al (1995) Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J Cell Biol 131(1):69–80
Daukas G, Zigmond SH (1985) Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. J Cell Biol 101(5 Pt 1):1673–1679
Dean DA, Strong DD, Zimmer WE (2005) Nuclear entry of nonviral vectors. Gene Ther 12(11):881–890
Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu Rev Biochem 78:857–902
Dos ST, Varela J, Lynch I et al (2011) Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines. PLoS ONE 6(9):e24438

Dowty ME, Williams P, Zhang G et al (1995) Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. Proc Natl Acad Sci USA 92(10):4572–4576
Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300(4):C723–C742
Ehrlich M, Boll W, van Oijen A et al (2004) Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 118(5):591–605
Fichter KM, Ingle NP, McLendon PM et al (2013) Polymeric nucleic acid vehicles exploit active interorganelle trafficking mechanisms. ACS Nano 7(1):347–364
Fletcher PA, Scriven DR, Schulson MN et al (2010) Multi-image colocalization and its statistical significance. Biophys J 99(6):1996–2005
Gabrielson NP, Pack DW (2009) Efficient polyethylenimine-mediated gene delivery proceeds via a caveolar pathway in HeLa cells. J Control Rel 136(1):54–61
Garcia GM, Alonso F, Chiva VA et al (1982) Phospholipid turnover during phagocytosis in human polymorphonuclear leucocytes. Biochem J 206(1):67–72
Geisow MJ (1984) Fluorescein conjugates as indicators of subcellular Ph—a critical-evaluation. Exp Cell Res 150(1):29–35
Georgieva JV, Kalicharan D, Couraud PO et al (2011) Surface characteristics of nanoparticles determine their intracellular fate in and processing by human blood-brain barrier endothelial cells in vitro. Mol Ther 19(2):318–325
Godbey WT, Wu KK, Mikos AG (1999) Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci USA 96(9):5177–5181
Gosselin MA, Guo W, Lee RJ (2001) Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. Bioconjug Chem 12(6):989–994
Gratton SE, Ropp PA, Pohlhaus PD et al (2008) The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci USA 105(33):11613–11618
Grosse S, Aron Y, Thevenot G et al (2007) Cytoskeletal involvement in the cellular trafficking of plasmid/PEI derivative complexes. J Control Rel 122(1):111–117
Grossman E, Medalia O, Zwerger M (2012) Functional architecture of the nuclear pore complex. Annu Rev Biophys 41:557–584
Gruenberg J, Stenmark H (2004) The biogenesis of multivesicular endosomes. Nat Rev Mol Cell Biol 5(4):317–323
Hansen SH, Sandvig K, van Deurs B (1993) Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J Cell Biol 121(1):61–72
Harris J, Werling D, Hope JC et al (2002) Caveolae and caveolin in immune cells: distribution and functions. Trends Immunol 23(3):158–164
Harush-Frenkel O, Rozentur E, Benita S et al (2008) Surface charge of nanoparticles determines their endocytic and transcytotic pathway in polarized MDCK cells. Biomacromolecules 9(2):435–443
Hasegawa S, Hirashima N, Nakanishi M (2001) Microtubule involvement in the intracellular dynamics for gene transfection mediated by cationic liposomes. Gene Ther 8(21):1669–1673
He B, Lin P, Jia Z et al (2013) The transport mechanisms of polymer nanoparticles in Caco-2 epithelial cells. Biomaterials 34(25):6082–6098
Heller A, Brockhoff G, Goepferich A (2012) Targeting drugs to mitochondria. Eur J Pharm Biopharm 82(1):1–18
Hemmerich PH, von Mikecz AH (2013) Defining the subcellular interface of nanoparticles by live-cell imaging. PLoS ONE 8(4):e62018
Henriques R, Griffiths C, Hesper RE et al (2011) PALM and STORM: unlocking live-cell super-resolution. Biopolymers 95(5):322–331
Hinshaw JE (2000) Dynamin and its role in membrane fission. Annu Rev Cell Dev Biol 16:483–519
Huang F, Khvorova A, Marshall W et al (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem 279(16):16657–16661
Hufnagel H, Hakim P, Lima A et al (2009) Fluid phase endocytosis contributes to transfection of DNA by PEI-25. Mol Ther 17(8):1411–1417

Ilina P, Hyvonen Z, Saura M et al (2012) Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems. J Control Release 163(3):385–395
Ivanov AI (2008) Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Method Mol Biol 440:15–33
Jones AT (2007) Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. J Cell Mol Med 11(4):670–684
Kanzaki M, Pessin JE (2002) Caveolin-associated filamentous actin (Cav-actin) defines a novel F-actin structure in adipocytes. J Biol Chem 277(29):25867–25869
Kerr MC, Teasdale RD (2009) Defining macropinocytosis. Traffic 10(4):364–371
Khalil IA, Kogure K, Akita H et al (2006a) Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev 58(1):32–45
Khalil IA, Kogure K, Futaki S et al (2006b) High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. J Biol Chem 281(6):3544–3551
Khalil IA, Kogure K, Futaki S et al (2008) Octaarginine-modified liposomes: enhanced cellular uptake and controlled intracellular trafficking. Int J Pharm 354(1–2):39–48
Kilsdonk EP, Yancey PG, Stoudt GW et al (1995) Cellular cholesterol efflux mediated by cyclodextrins. J Biol Chem 270(29):17250–17256
Kim AJ, Boylan NJ, Suk JS et al (2012) Non-degradative intracellular trafficking of highly compacted polymeric DNA nanoparticles. J Control Rel 158(1):102–107
Kirchhausen T (1999) Adaptors for clathrin-mediated traffic. Annu Rev Cell Dev Biol 15:705–732
Larsen JD, Ross NL, Sullivan MO (2012) Requirements for the nuclear entry of polyplexes and nanoparticles during mitosis. J Gene Med 14(9–10):580–589
Lechardeur D, Verkman AS, Lukacs GL (2005) Intracellular routing of plasmid DNA during non-viral gene transfer. Adv Drug Deliv Rev 57(5):755–767
Lemmon SK (2001) Clathrin uncoating: Auxilin comes to life. Curr Biol 11(2):R49–R52
Lencer WI, Hirst TR, Holmes RK (1999) Membrane traffic and the cellular uptake of cholera toxin. Biochim Biophys Acta 1450(3):177–190
Leroueil PR, Hong S, Mecke A et al (2007) Nanoparticle interaction with biological membranes: does nanotechnology present a Janus face? Acc Chem Res 40(5):335–342
Li W, Chen L, Huang Z et al (2011) The influence of cyclodextrin modification on cellular uptake and transfection efficiency of polyplexes. Org Biomol Chem 9(22):7799–7806
Liao JK, Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89–118
Luetterforst R, Stang E, Zorzi N et al (1999) Molecular characterization of caveolin association with the golgi complex: identification of a cis-golgi targeting domain in the caveolin molecule. J Cell Biol 145(7):1443–1459
Lundberg M, Johansson M (2001) Is VP22 nuclear homing an artifact? Nat Biotechnol 19(8):713–714
Luzio JP, Pryor PR, Bright NA (2007) Lysosomes: fusion and function. Nat Rev Mol Cell Biol 8(8):622–632
Ma Y, Greener T, Pacold ME et al (2002) Identification of domain required for catalytic activity of Auxilin in supporting clathrin uncoating by Hsc70. J Biol Chem 277(51):49267–49274
Manders EM, Stap J, Brakenhoff GJ et al (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J Cell Sci 103(3):857–862
Massignani M, LoPresti C, Blanazs A et al (2009) Controlling cellular uptake by surface chemistry, size, and surface topology at the nanoscale. Small 5(21):2424–2432
Maxfield FR, McGraw TE (2004) Endocytic recycling. Nat Rev Mol Cell Biol 5(2):121–132
McNally JG, Karpova T, Cooper J et al (1999) Three-dimensional imaging by deconvolution microscopy. Methods 19(3):373–385
Mercer J, Helenius A (2009) Virus entry by macropinocytosis. Nat Cell Biol 11(5):510–520

Mettlen M, Platek A, Van Der Smissen P et al (2006) Src triggers circular ruffling and macropinocytosis at the apical surface of polarized MDCK cells. Traffic 7(5):589–603
Moreno-Ruiz E, Galan-Diez M, Zhu W et al (2009) Candida albicans internalization by host cells is mediated by a clathrin-dependent mechanism. Cell Microbiol 11(8):1179–1189
Nakai T, Kanamori T, Sando S et al (2003) Remarkably size-regulated cell invasion by artificial viruses. Saccharide-dependent self-aggregation of glycoviruses and its consequences in glycoviral gene delivery. J Am Chem Soc 125(28):8465–8475
Nakase I, Niwa M, Takeuchi T et al (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol Ther 10(6):1011–1022
Ogris M, Wagner E, Steinlein P (2000) A versatile assay to study cellular uptake of gene transfer complexes by flow cytometry. Biochim Biophys Acta 1474(2):237–243
Paine PL, Moore LC, Horowitz SB (1975) Nuclear envelope permeability. Nature 254(5496):109–114
Pante N, Kann M (2002) Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. Mol Biol Cell 13(2):425–434
Parton RG, Joggerst B, Simons K (1994) Regulated internalization of caveolae. J Cell Biol 127(5):1199–1215
Parton RG, Richards AA (2003) Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic 4(11):724–738
Parton RG, Simons K (2007) The multiple faces of caveolae. Nat Rev Mol Cell Biol 8(3):185–194
Pearson H (2007) The good, the bad and the ugly. Nature 447(7141):138–140
Pelkmans L, Kartenbeck J, Helenius A (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol 3(5):473–483
Perumal OP, Inapagolla R, Kannan S et al (2008) The effect of surface functionality on cellular trafficking of dendrimers. Biomaterials 29(24–25):3469–3476
Phizicky E, Bastiaens PI, Zhu H et al (2003) Protein analysis on a proteomic scale. Nature 422(6928):208–215
Pollard H, Remy JS, Loussouarn G et al (1998) Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J Biol Chem 273(13):7507–7511
Rejman J, Oberle V, Zuhorn IS et al (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 377(Pt 1):159–169
Roberts RL, Nath J, Friedman MM et al (1982) Effects of taxol on human neutrophils. J Immunol 129(5):2134–2141
Rodal SK, Skretting G, Garred O et al (1999) Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol Biol Cell 10(4):961–974
Ros-Baro A, Lopez-Iglesias C, Peiro S et al (2001) Lipid rafts are required for GLUT4 internalization in adipose cells. Proc Natl Acad Sci USA 98(21):12050–12055
Rothberg KG, Ying YS, Kolhouse JF et al (1990) The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. J Cell Biol 110(3):637–649
Ruthardt N, Brauchle C (2010) Visualizing uptake and intracellular trafficking of gene carriers by single-particle tracking. Top Curr Chem 296:283–304
Saftig P, Klumperman J (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat Rev Mol Cell Biol 10(9):623–635
Sakhrani NM, Padh H (2013) Organelle targeting: third level of drug targeting. Drug Des Dev Ther 7:585–599
Salh B, Wagey R, Marotta A et al (1998) Activation of phosphatidylinositol 3-kinase, protein kinase B, and p70 S6 kinases in lipopolysaccharide-stimulated Raw 264.7 cells: differential effects of rapamycin, Ly294002, and wortmannin on nitric oxide production. J Immunol 161(12):6947–6954

Salvati A, Aberg C, dos Santos T et al (2011) Experimental and theoretical comparison of intracellular import of polymeric nanoparticles and small molecules: toward models of uptake kinetics. Nanomedicine 7(6):818–826
Sandvig K, Olsnes S, Petersen OW et al (1987) Acidification of the cytosol inhibits endocytosis from coated pits. J Cell Biol 105(2):679–689
Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem 66:511–548
Schnitzer JE, Allard J, Oh P (1995) NEM inhibits transcytosis, endocytosis, and capillary permeability: implication of caveolae fusion in endothelia. Am J Physiol 268(2):H48–H55
Scriven DR, Lynch RM, Moore ED (2008) Image acquisition for colocalization using optical microscopy. Am J Physiol Cell Physiol 294(5):C1119–C1122
Shi J, Chou B, Choi JL et al (2013) Investigation of polyethylenimine/DNA polyplex transfection to cultured cells using radiolabeling and subcellular fractionation methods. Mol Pharm 10(6):2145–2156
Smart EJ, Ying YS, Conrad PA et al (1994) Caveolin moves from caveolae to the golgi apparatus in response to cholesterol oxidation. J Cell Biol 127(5):1185–1197
Spoden G, Freitag K, Husmann M et al (2008) Clathrin- and caveolin-independent entry of human papillomavirus type 16–involvement of tetraspanin-enriched microdomains (TEMs). PLoS ONE 3(10):e3313
Stoorvogel W, Strous GJ, Geuze HJ et al (1991) Late endosomes derive from early endosomes by maturation. Cell 65(3):417–427
Suh J, An Y, Tang BC et al (2012) Real-time gene delivery vector tracking in the endo-lysosomal pathway of live cells. Microsc Res Tech 75(5):691–697
Suh J, Wirtz D, Hanes J (2003) Efficient active transport of gene nanocarriers to the cell nucleus. Proc Natl Acad Sci USA 100(7):3878–3882
Thorek DL, Tsourkas A (2008) Size, charge and concentration dependent uptake of iron oxide particles by non-phagocytic cells. Biomaterials 29(26):3583–3590
Treuel L, Jiang X, Nienhaus GU (2013) New views on cellular uptake and trafficking of manufactured nanoparticles. J Royal Soc Interface 10(82):20120939
van der Aa MA, Huth US, Hafele SY et al (2007) Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. Pharm Res 24(8):1590–1598
Vaughan EE, Dean DA (2006) Intracellular trafficking of plasmids during transfection is mediated by microtubules. Mol Ther 13(2):422–428
Veiga E, Cossart P (2005) Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. Nat Cell Biol 7(9):894–900
Vercauteren D, Rejman J, Martens TF et al (2012) On the cellular processing of non-viral nanomedicines for nucleic acid delivery: mechanisms and methods. J Control Rel 161(2):566–581
Vercauteren D, Vandenbroucke RE, Jones AT et al (2010) The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. Mol Ther 18(3):561–569
Vida T, Gerhardt B (1999) A cell-free assay allows reconstitution of Vps33p-dependent transport to the yeast vacuole/lysosome. J Cell Biol 146(1):85–98
Villanueva A, Canete M, Roca AG et al (2009) The influence of surface functionalization on the enhanced internalization of magnetic nanoparticles in cancer cells. Nanotechnology 20(11):115103
Visser CC, Stevanovic S, Heleen VL et al (2004) Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells in vitro. J Drug Target 12(3):145–150
von Gersdorff K, Sanders NN, Vandenbroucke R et al (2006) The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther 14(5):745–753
Wang AZ (2012) Nanoparticle drug delivery: focusing on the therapeutic cargo. Nanomedicine (Lond.) 7(10):1463–1465

Wang G, Norton AS, Pokharel D et al (2013) KDEL peptide gold nanoconstructs: promising nanoplatforms for drug delivery. Nanomedicine 9(3):366–374

Wang LH, Rothberg KG, Anderson RG (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol 123(5):1107–1117

Wang Z, Tiruppathi C, Minshall RD et al (2009) Size and dynamics of caveolae studied using nanoparticles in living endothelial cells. ACS Nano 3(12):4110–4116

Wasungu L, Hoekstra D (2006) Cationic lipids, lipoplexes and intracellular delivery of genes. J Control Rel 116(2):255–264

West MA, Bretscher MS, Watts C (1989) Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. J Cell Biol 109(1):2731–2739

Westermann M, Steiniger F, Richter W (2005) Belt-like localisation of caveolin in deep caveolae and its re-distribution after cholesterol depletion. Histochem Cell Biol 123(6):613–620

Yumoto R, Nishikawa H, Okamoto M et al (2006) Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN. Am J Physiol Lung Cell Mol Physiol 290(5):L946–L955

Zaki NM, Tirelli N (2010) Gateways for the intracellular access of nanocarriers: a review of receptor-mediated endocytosis mechanisms and of strategies in receptor targeting. Expert Opin Drug Deliv 7(8):895–913

Zauner W, Farrow NA, Haines AM (2001) In vitro uptake of polystyrene microspheres: effect of particle size, cell line and cell density. J Control Rel 71(1):39–51

Zhao F, Zhao Y, Liu Y et al (2011) Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. Small 7(10):1322–1337

Zuidam NJ, Posthuma G, de Vries ET et al (2000) Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. J Drug Target 8(1):51–66