Chapter 10
Investigating Interactions Between Nanoparticles and Cells: Internalization and Intracellular Trafficking

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Abstract Nanoparticles used as drug nanocarriers offer unique possibilities to overcome cellular barriers in order to improve the delivery of various molecules, including biomacromolecules such as nucleic acids or proteins. Depending on nanoparticle characteristics and the type of cells considered, various mechanisms of internalization may occur, as well as subsequent intracellular trafficking pathways. Understanding these pathways may have important pharmacological implications. This chapter will review the main nanoparticle internalization and trafficking mechanisms and their experimental characterizations, allowing to understand how they are affected by nanoparticle physicochemical properties. The phagocytosis pathway will first be described, being increasingly well characterized and understood, which has allowed several successes in the treatment of some cancers and infectious diseases. In contrast, other non-phagocytic pathways encompass various complex mechanisms, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis. Although more challenging to control for pharmaceutical drug delivery applications, they are actively investigated in order to tailor nanocarriers able to deliver anticancer agents, nucleic acids, proteins, and peptides for therapeutic applications.

Keywords Caveolae · Caveolae-mediated endocytosis · Clathrin · Clathrin-mediated endocytosis · Endocytosis · Endosome · Internalization · Intracellular trafficking · Lysosome · Macropinocytosis · Opsonization · Phagocytosis · Pinocytosis · Receptor-mediated endocytosis

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1 Introduction

In order to be active, a drug has to reach the relevant pharmacological target, which means undergoing a complex series of interactions with the cells of the body. When this target is located inside diseased cells, the therapeutic molecule must generally: (1) cross one or various biological membranes (e.g., mucosa, epithelium, endothelium) before (2) reaching the target cell and diffusing through the plasma membrane to (3) finally gain access to the appropriate organelle where the biological target is located. Deviating from this ideal path may decrease the drug efficiency but also entail side effects and toxicity.

More than 30 years ago, the idea emerged to tailor carriers small enough to ferry the active substance to the target cell and its relevant subcellular compartment. In the seventies, the proof of concept has been done that submicronic lipid vesicles known as liposomes (Black and Gregoriadis 1974), as well as synthetic polymer nanoparticles (Couvreur et al. 1977), were able to concentrate into cells, molecules that did not diffuse intracellularly. It became clear that such 'nanocarriers' had a great potential for the targeted delivery of drugs. Today, such approach is exploited to optimize the intracellular delivery of many small molecules, but also macromolecules like nucleic acids, peptides, or proteins.

Depending on nanoparticle characteristics and the type of cells considered, various mechanisms of internalization may occur, as well as subsequent intracellular trafficking pathways. Understanding these pathways may have important pharmacological implications. This chapter will review the main nanoparticle internalization and trafficking mechanisms and their experimental characterizations, allowing to understand how they are affected by nanoparticle physicochemical properties. The phagocytosis pathway will first be described, being increasingly well characterized and understood, which has allowed several successes in the treatment of some cancers and infectious diseases. In contrast, other non-phagocytic pathways encompass various complex mechanisms, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis. The intracellular fate will vary accordingly, which has important implications for intracellular drug delivery.

2 Nanoparticle Internalization and Trafficking Routes Occurring in Cells

Depending on the nature of the cells concerned, and the physicochemical properties of the nanocarriers, different endocytosis modes may occur (Fig. 1): phagocytosis or other endocytic routes, i.e., mainly macropinocytosis, clathrin- and caveolae-mediated endocytosis.

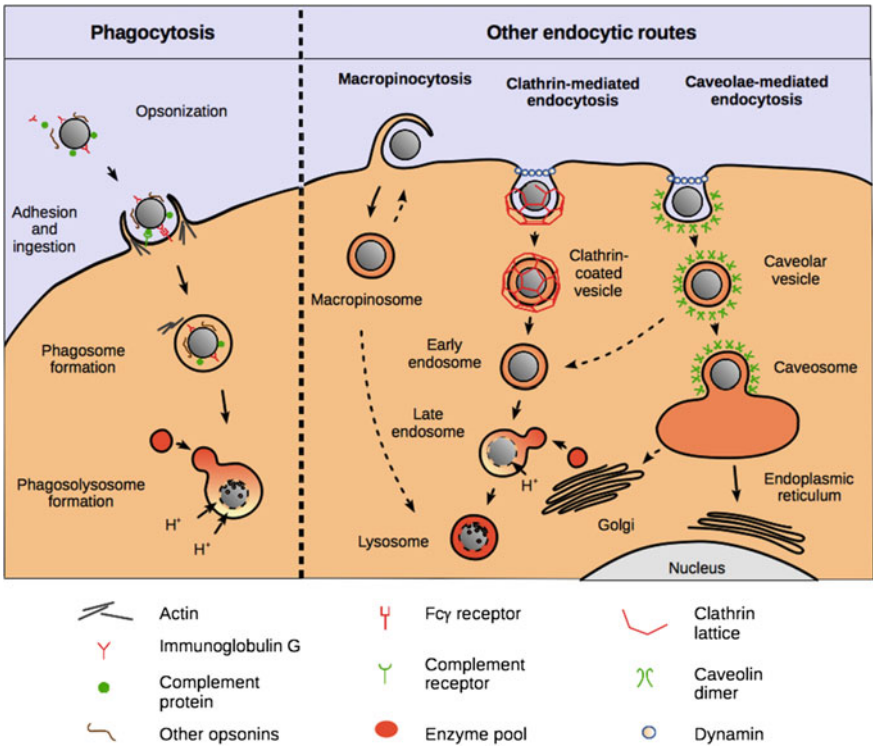

Fig. 1 Overview of the major internalization pathways of nanoparticles by cells, and corresponding trafficking routes

2.1 Phagocytosis

Phagocytosis occurs mostly in specialized cells, also called professional phagocytes: macrophages, monocytes, neutrophils, and dendritic cell (Aderem and Underhill 1999). In particular, macrophages of the reticuloendothelial system (RES, also known as mononuclear phagocytic system) are the major phagocytes involved in the uptake of nanoparticles and have been by far the most studied for drug delivery purposes.

The phagocytic pathway of entry into cells can be described using three distinct steps:

1. Recognition by opsonization in the bloodstream. Opsonization is an important process occurring before the phagocytosis itself. It consists in tagging the foreign nanoparticles by proteins called opsonins, making the former visible to macrophages. This typically takes place in the bloodstream rapidly after introduction of the particles (Alexis et al. 2008). Major opsonins include immunoglubulins (Ig) G (and M) as well as complement components (C3, C4,

C5) (Frank and Fries 1991; Vonarbourg et al. 2006), in addition to other blood serum proteins (including laminin, fibronectin, C-reactive protein, type-I collagen) (Owens and Peppas 2006).

2. Opsonized particles then attach to macrophage surface through specific receptor-ligand interactions. The major and best-studied receptors for this purpose include the Fc receptors (FcR) and the complement receptors (CR). FcRs bind to the constant fragment of particle-adsorbed immunoglobulins, while CRs mostly bind to C3 fragments (Aderem and Underhill 1999; Groves et al. 2008). Other receptors including the mannose/fructose and scavengers receptors can be involved in the phagocytosis (Aderem and Underhill 1999). Receptor ligation is the beginning of a signaling cascade mediated by Rho-familiy GTPases (Caron and Hall 1998) which triggers actin assembly, forming pseudopodia that zipper up around the particle to engulf it.
3. The resulting phagosome, having a minimum size of 250 nm (Alberts et al. 2002), ferries the particle throughout the cytoplasm. As actin is depolymerized from the phagosome, the newly denuded vacuole membrane becomes accessible to early endosomes (Swanson and Baer 1995). Through a series of fusion and fission events, the vacuolar membrane and its contents mature, fusing with late endosomes and ultimately lysosomes to form a phagolysosome. The rate of these events depends on the surface properties of the ingested particle, typically from half to several hours (Aderem and Underhill 1999). The phagolysosomes acidify and acquire many enzymes, including esterases and cathepsins (Claus et al. 1998), leading to the degradation of biodegradable or bioerodible particle material.

2.2 Other Endocytic Pathways

The non-phagocytic endocytosis has been traditionally referred to as pinocytosis, literally ‘cell drinking’, i.e., uptake of fluids and solutes, as opposed to ‘cell eating’, i.e., uptake of solid particles for phagocytosis. This terminology may not be relevant for the study of the nanoparticle-cell interaction, since solid particles, due to their small size, can be internalized through these non-phagocytic pathways. Unlike phagocytosis, restricted to specialized cells, other endocytic pathways occur in virtually all cells by four main mechanisms: clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and other clathrin- and caveolae-independent endocytosis.

2.2.1 Clathrin-Mediated Endocytosis

Endocytosis via clathrin-coated pits, or clathrin-mediated endocytosis (CME), occurs constitutively in all mammalian cells, and fulfills crucial physiological roles, including nutrient uptake and intracellular communication (Schmid 1997; Di Fiore

and De Camilli 2001). For most cell types, CME serves as the main mechanism of internalization for macromolecules and plasma membrane constituents. CME via specific receptors-ligand interaction is the best-described mechanism (to such extent that it was previously referred to as receptor-mediated endocytosis (RME)); however, it is now clear that alternative nonspecific endocytosis via clathrin-coated pits also exists (as well as receptor-mediated but clathrin-independent endocytosis). The CME internalization pathway (either receptor-dependent or independent) is associated to degradation of the endocyted material in lysosomes. This should be taken into account with respect to degradation of the nanocarrier but also of fragile cargo molecules.

Receptor-dependent CME is one of the best-characterized endocytic mechanisms. It is a shared pathway for the internalization of a variety of ligand-receptor complexes (Mukherjee et al. 1997). Many ligands have been used for this purpose, including low-density lipoprotein (LDL), transferrin, epidermal growth factor (EGF) (Bareford and Swaan 2007; Chavanpatil et al. 2006).

The endocytosis typically occurs in a membrane region enriched in the clathrin protein. The formation of an endocytosis vacuole is driven by assembly of a basket-like structure (Kanaseki and Kadota 1969) formed by polymerization of clathrin units, which deforms the membrane into a coated pit. Some receptors, like the LDL receptors, are concentrated in these clathrin-coated pits, while others receptors like the transferrin and EGF receptors become concentrated upon ligand binding (Mukherjee et al. 1997). The fission of the vesicle, requiring the GTPase dynamin, leads to so-called clathrin-coated vesicles. The uncoating of the vesicles later allows the recycling of clathrin units (Conner and Schmid 2003). Some ligands are also recycled, as transferrin and riboflavin (Bareford and Swaan 2007). The resulting endocytic vesicle may have an average size of 100 (Bareford and Swaan 2007) or 120 nm (Conner and Schmid 2003). This vesicle delivers its cargo to early endosomes, which are acidified (pH $\sim$6) (Al-Awqati 1986). Some receptors and ligands dissociate at this stage and are recycled for another round of delivery (e.g., LDL receptor, transferrin and its receptor). The early endosomes then mature into late endosomes (pH $\sim$5) which, after fusion with prelysosomal vesicles containing acid hydrolases, generate a harsh environment prone to degradation of the internalized cargo (Mukherjee et al. 1997; Bareford and Swaan 2007).

In the case of polarized cells, the recycled molecules can either return to the membrane from which they were internalized, or they can cross the cell and be delivered to the opposite membrane in a process called transcytosis (Matter and Mellman 1994). Transcytosis of transferrin is of particular importance in the case if endothelial cells forming the blood–brain barrier (BBB) (Jones and Shusta 2007).

Another CME mechanism, involving nonspecific adsorptive pinocytosis, has been simply referred to as fluid-phase endocytosis by some authors (Sun et al. 2004). Compounds absorbed by this pathway avoid direct binding with membrane constituents, but often display nonspecific charges and hydrophobic interactions with the cell membrane. Fluid entry occurs via clathrin-coated vesicles as described above, internalizing also receptor-ligands located in these pits, together with extracellular fluid and its content (Bareford and Swaan 2007). Apart from the

different modes of interaction with the membrane, the major specificity of this pathway is a slower internalization rate compared to the receptor-dependent CME (Strømhaug et al. 1997).

2.2.2 Caveolae-Mediated Endocytosis

Although CME is the predominant endocytosis mechanism in most cells, alternative pathways have been also identified, in particular the caveolae-mediated endocytosis (CvME). Caveolae are characteristic flask-shaped membrane invaginations, having a size generally reported in the lower end of the 50–100 nm range (Mukherjee et al. 1997; Bareford and Swaan 2007; Conner and Schmid 2003; van Oss 1978), typically 50–80 nm. They are lined by caveolin, a dimeric protein, and enriched with cholesterol and sphingolipids (Mayor and Pagano 2007). Caveolae are particularly abundant in endothelial cells, where they can constitute 10–20 % of the cell surface (Conner and Schmid 2003), but also smooth muscle cells and fibroblasts (Chen et al. 1997). CvME are involved in endocytosis and trancytosis of various proteins; they also constitute a port of entry for viruses (typically the SV40 virus) (Cheng et al. 2006), and receive increasing attention for drug delivery applications using nanocarriers.

Unlike CME, CvME is a highly regulated process, involving complex signaling, which may be driven by the cargo itself (Bareford and Swaan 2007; Conner and Schmid 2003). After binding to the cell surface, particles move along the plasma membrane to caveolae invaginations, where they may be maintained through receptor-ligand interactions (Bareford and Swaan 2007). The fission of caveolae from the membrane, mediated by the GTPase dynamin (Parton and Simons 2007), then generates the cytosolic caveolar vesicle which does not contain any enzymatic cocktail (this pathway is employed by many pathogens to escape degradation by lysosomal enzymes) (Chen et al. 1997). The use of nanocarriers exploiting CvME may therefore be advantageous to by-pass the lysosomal degradation pathway when the carried drug (e.g., peptides, proteins, nucleic acids, etc.) is highly sensitive to enzymes.

On the whole, the uptake kinetics of CvME is known to occur at a much slower rate than that of CME (Marsh and Helenius 2006). Ligands known to be internalized by CvME include folic acid (Oh et al. 1998), albumin (Rejman et al. 2005) and cholesterol (Bareford and Swaan 2007).

2.2.3 Macropinocytosis

Macropinocytosis is another type of clathrin-independent endocytosis pathway (Chang et al. 1992), occurring in many cells including macrophages (Mukherjee et al. 1997). It accompanies the membrane ruffling and is induced especially upon stimulation by growth factors or other signals (Mukherjee et al. 1997; Conner and Schmid 2003). Macropinocytosis occurs via the formation of actin-driven membrane protrusions, similarly to phagocytosis. However, in this case, the protrusions

do not zipper up along the ligand-coated particle; instead, they collapse onto and fuse with the plasma membrane (Conner and Schmid 2003). This generates large endocytic vesicles, called macropinosomes, which sample the extracellular milieu and have a size generally higher than 1 µm (Conner and Schmid 2003) [and sometimes as large as 5 µm (Mukherjee et al. 1997)]. The intracellular fate of macropinosomes varies depending on the cell type, but in most cases, they acidify and shrink. They may eventually fuse with lysosomal compartments or recycle their content to the surface (Mukherjee et al. 1997). Macropinosomes have not been reported to contain any specific coating, nor to concentrate receptors (Schnitzer 2001). This endocytic pathway does not seem to display any selectivity, but is involved, among others, in the uptake of drug nanocarriers.

2.2.4 Other Endocytic Pathways

Various clathrin- and caveolae-independent endocytosis have also been described. In particular, pathways similar to CvME involving cholesterol-rich microdomains called 'rafts', having a 40–50 nm diameter, have received increasing attention (Conner and Schmid 2003). A classification for the clathrin- and caveolae-independent pathways has been proposed (van Oss 1978). However, the understanding of their implications in the interactions with drug delivery nanosytems is still in a nascent stage.

3 Investigation of Nanoparticle Uptake and Internalization

3.1 Phagocytosis

3.1.1 Experimental Techniques

Nanoparticles are mainly characterized by (1) their size, measured directly by various microscopy techniques or indirectly by dynamic light scattering; (2) their surface properties such as charge, indirectly evaluated from their zeta potential in a reference electrolyte, hydrophilicity/hydrophobicity and chemical composition (chromatography and surface spectroscopy); (3) their morphology, assessed by microscopy. The visualization of their interaction with cells can be achieved through incorporation of a fluorescent tracer which can be either covalently bound to the nanocarriers or to the encapsulated drug or simply incorporated into the nanoparticles (simulating the encapsulated drug). Confocal microscopy has shown invaluable usefulness to monitor the intracellular trafficking, for example when performed on unfixed living macrophages (Swanson and Watts 1995). The quantification of phagocytosis can be precisely performed using radiolabeled polymers (in case of nanoparticles) or lipids (in case of liposomes) as well as radiolabeled drugs, and in some cases through an extraction and chromatography process. The

characterization of phagocytosis as an active internalization pathway is often achieved through inhibition by lowering temperature or using metabolic inhibitors (e.g, sodium azide) or inhibitors of actin polymerization (e.g, cytochalasin D). The modern tools of proteomics and live imaging are also used to characterize the molecular mechanisms of phagocytosis (Groves et al. 2008). Finally, the complement activation induced by the nanocarrier's surface can be investigated by measuring the residual hemolytic capacity of the serum after incubation with the particles (Racoosin and Swanson 1992).

3.1.2 Nanocarrier Characteristics Influencing Phagocytosis

Size

Although a minimum size of 0.5 μm for a particle to undergo phagocytosis is often put forward (Aderem and Underhill 1999; Groves et al. 2008), this statement is not always (Martina et al. 2007) justified and generally used to highlight the wide size tolerance for phagocytosis ['macrophages can eat bigger than their head' (Labarre et al. 1993)] compared to other endocytosis modes. Model polystyrene particles in a range of around 250 nm to 3 μm have actually been shown to have an optimal in vitro phagocytosis rate (particle weight per cell), merely increasing with the particle size (the number of particles by cell decreases by three orders of magnitude at the same time), whereas nanoparticles smaller than 250 nm were less efficiently internalized (wt/cell) (Desjardins and Griffiths 2003; Aderem 2002). Similarly, particles based on other polymers [human serum albumin (Korn and Weisman 1967), modified cellulose (Roberts and Quastel 1963; Schäfer et al. 1992), poly (methylmethacrylate) (Korn and Weisman 1967; Tabata and Ikada 1988) and poly (alkylcyanoacrylate) (PACA) (Korn and Weisman 1967)] exhibited a higher uptake when their size increased from around 200 nm to several microns. However, in the case of drug carriers intended for an intravenous administration (requiring small particle size to avoid embolization), a size of 200 nm can be considered as optimal (Korn and Weisman 1967). Liposomes generally display the same pattern: larger (>100 nm) and multilamelar ones are less numerous but deliver a higher payload to macrophages, compared to the smaller (and unilamelar) ones (Tabata and Ikada 1990; Müller et al. 1992; Rahman et al. 1982; Schwendener et al. 1984; Harashima et al. 1995). Some studies reported a more balanced (Moghimi and Szebeni 2003) or even opposite (Rudt and Muller 1993) impact of the liposome size, suggesting that other factors like surface properties may get the upper hand.

In the presence of serum, the size of the nanocarriers was observed to have a strong influence on the opsonin adsorption, and therefore on the phagocytosis. Indeed, the in vitro consumption of complement proteins was demonstrated to increase with the size of lipid nanocapsules (the total surface exhibited by the particles being constant) (Heath et al. 1985). This was explained by the fact that on the most curved surface of the smallest particles, the proper geometric configuration for efficient complement activation could be achieved less easily than on larger ones

(Vonarbourg et al. 2006). Noteworthy, in vitro and in vivo studies on 200–800 nm liposomes suggested two kinds of uptake by phagocytes: a size-dependant one involving opsonization, increasing with the size of liposomes, and a size-independent one, corresponding to unopsonized particles (Allen et al. 1991).

Finally, it is worth mentioning that some macrophages have shown a decrease in the phagocytosis of small particles (200 nm) and a shift to other endocytosis pathways like clathrin-mediated endocytosis (Vonarbourg et al. 2006). But in general, a low uptake by macrophages is observed for nanoparticles smaller than 100 nm, whatever the mechanism involved (Vonarbourg et al. 2006).

Surface Properties

Early studies performed on nanocarriers shown that liposomes (Harashima et al. 1994; Koval et al. 1998) as well as polymer nanoparticles (Gregoriadis 1978; Torchilin et al. 1980; Grislain et al. 1983) are rapidly cleared from the bloodstream by macrophages of the RES, virtually irrespective of the particle composition. Indeed the presence or not of a proper surface coating able to repel opsonins proved to be the bottom line in entering the phagocytosis pathway or not. The physico-chemical characteristics of the particle surface are thus critical and determine the interaction with opsonins prior to cell surface.

The most important driving forces for protein adsorption are often regarded to be ionic and hydrophobic interactions (combined with entropic gain caused by conformational changes of the protein during adsorption) (Lenaerts et al. 1984; Kreuter et al. 1979). In the case of nanocarriers, highly charged particles have proven to fix complement proteins, especially liposomes (Claesson et al. 1995; Verrecchia et al. 1993), either negatively or positively charged, whatever the complement activation pathway (Devine et al. 1994; Gabizon and Papahadjopoulos 1992). The observation that the former often activate even more complement than the latter may originate in differences in the amount of adsorbed proteins and the opsonins/dysopsonins ratios (Juliano and Stamp 1975; Chonn et al. 1991) (dysopsonins decrease recognition by phagocytes). More specifically, apolipoproteins have been proposed to contribute specifically to the uptake by hepatocyte (Juliano and Lin 1980). In the case of polymer nanoparticles, although negative charges can be related to a higher uptake (Vonarbourg et al. 2006; Moghimi et al. 1993; Scherphof and Kamps 1998), the surface hydrophobicity appears to be the key factor for opsonization. Nanoparticles prepared from hydrophobic polymers poly(styrene) (Mosqueira et al. 1999), poly (lactide) (PLA) (Roser et al. 1998; Norman et al. 1992), poly(lactide-*co*-glycolide) (PLGA) (Leroux et al. 1994) and poly(alkylcyanoacrylate) (PACA) (Leroux et al. 1995) undergo important adsorption of Ig, complement proteins and other plasma proteins like albumin, either in vitro or in vivo. To account for these observations, a higher level of protein adsorption on hydrophobic surfaces than on hydrophilic ones has been proposed (Esmaeili et al. 2008; Bertholon et al. 2006), as well as high affinity of IgG and albumin for hydrophobic regions (Gabizon and Papahadjopoulos 1992; Jeon et al. 1991).

However, as a general rule, it is considered that the in vivo fate of exogenous nanoparticles is opsonization and phagocytosis by RES's protagonists, be they liposomes or polymer nanoparticles, with little discrimination regarding their composition, unless the particles possess a very small size (lower than 50–100 nm) or, more importantly, a specific hydrophilic coating able to repel opsonins. Poly (ethylene glycol) [PEG, also known as poly(oxyethylene)] has been extensively described for this purpose (Vonarbourg et al. 2006; Owens and Peppas 2006). In general, PEGylated nanocarriers dramatically decreased the in vitro opsonin adsorption and macrophage uptake, as compared to their non-PEGylated counterparts. After intravenous administration, PEGylation results in a decreased RES uptake and a prolonged circulation half-life, from typically few minutes to several hours. Besides PEG, polysaccharides have also been proposed as alternative hydrophilic polymers (Ilium et al. 1986).

When opsonized nanocarriers encounter macrophage surface, it is noteworthy that polymer nanoparticles and liposomes, whose structure and chemical composition strongly differ, still show similar interactions with macrophages, based on their surface electric charge. Liposomes displaying a negatively charged surface, generally containing the negatively charged phospholipids phosphatidylserine and phosphatidylglycerol, have a much higher binding to and phagocytosis by macrophages as compared to neutral vesicles (Moghimi and Szebeni 2003; Cullis et al. 1998; Labarre 2012; Lee et al. 1992a, b, 1993); the same is true for positively charged liposomes (Tabata and Ikada 1990; Müller et al. 1992). A similar pattern was found for negatively and positively charged polymer nanoparticles compared to neutral ones (Roberts and Quastel 1963; Schäfer et al. 1992; Moghimi et al. 1993; Scherphof and Kamps 1998). Additionally, hydrophobic nanoparticles are more readily captured than hydrophilic nonionic ones (Roberts and Quastel 1963). Several mechanisms have been proposed to account for the preferential uptake of charged particles: existence of high charge density areas at the cell surface able to mediate endocytosis of positively charged particles (Cullis et al. 1998; Hsu and Juliano 1982) and involvement of nonspecific interactions with nonspecific receptors by electrostatic interactions for negative particles (Müller et al. 1992), especially with type B scavenger receptor (Raz et al. 1981). Taking advantage of macrophage receptors to enhance phagocytosis has been further achieved by coupling specific ligands to nanocarriers. For example, grafting rabbit Ig (Větvicka and Fornůsek 1987) as well as mouse monoclonal antibody (Rigotti et al. 1995) to liposomes greatly enhanced their uptake by rat and human macrophages, respectively, most probably through increased FcR binding. Mannose receptors have also been exploited by the introduction of mannose residue and neoglycoprotein on liposome surface, enhancing uptake by murine Kupffer cells and peritoneal macrophages (Derksen et al. 1988; Betageri et al. 1993; Barratt et al. 1986). Other ways have also being explored, as attested by a study on the plasma membrane glycoprotein CD44 and the possible involvement of scavenger receptors (Muller and Schuber 1989). However, if the aforementioned studies provide decisive insight in the nanoparticle surface—macrophage interaction, it should be kept in mind that, as

soon as in vivo is concerned (or even in vitro studies in presence of serum), the opsonization process is likely to take precedence.

Shape

The vast majority of nanoparticles developed for drug delivery have a spherical shape. However, the control of particle shape is receiving increasing attention in order to control phagocytosis. First, maintaining or not the particle's spherical shape, i.e, rigidity, can be a significant factor. As far as interaction with the cell membrane is concerned, macrophages tend to show a strong preference for rigid particles. One study showed that soft polyacrylamide particles were unable to stimulate the assembly of actin filaments required for the formation and closure of phagosomes, as opposed to rigid particles (having the same total polymer mass and surface properties) (Kole et al. 1994). On the other hand, particle rigidity can have an opposite effect on opsonization. Rigid liposome membranes, composed of cholesterol and saturated phospholipids with a high melting point, are indeed known to decrease complement activation and thus phagocytosis (Rudt and Muller 1993; Jeon et al. 1991). Similarly, core-shell nanoparticles having a rigid polystyrene core were significantly less prone to uptake by RES than nanoparticles made of a more flexible core, based on fluid-like poly(methyl acrylate) (PMA) (Yu et al. 1997): a flexible particle is thought to provide a greater number of surface interactions with the biological environment (Alexis et al. 2008). Thus, no clear relationship emerges between nanocarrier rigidity and phagocytosis.

Besides particle rigidity, other works have focused on the control of the shape itself. The rationale behind this approach can be found in the well-known exposure of macrophages to exogenous particles of a high geometrical variety, like rod-shaped bacteria, *Escherichia coli* and *Bacillus anthracis*, disk-shaped senescent erythrocytes or multiple culpate and solpate airborne pollen grains. Advances in the synthesis techniques now allow a more precise control of particle geometry (Beningo and Wang 2002; Sun et al. 2005). Lipid disks have been developed as alternatives to liposomes, having a diameter of 120 nm (Champion et al. 2007) to 250 nm (Champion et al. 2007), and a thickness of only a few nanometers, showing efficient uptake by RES macrophages. Other lipid assemblies, either bilayer disks (Guo 2001) or cube-shaped so-called cubosomes (Larabi et al. 2003) have been proposed as new drug nanocarriers. However, the impact of the shape of such systems on phagocytosis as compared to liposomes remains to be fully elucidated. As demonstrated for polystyrene particles of various shapes (ellipsoids, disks, UFO-like), the local particle shape at the point of contact dictates whether macrophages initiate phagocytosis or simply spread on particles (Carmona-Ribeiro 2006). For example, a macrophage attached to an ellipse at the pointed end will internalize it in a few minutes, while a macrophage attach to a flat region of the same ellipse will not internalize it for over 12 h. This effect, originating in the complexity of actin structure required to initiate uptake, was even prevailing on particle size (Carmona-Ribeiro 2006).

3.1.3 Theoretical Models of Nanocarrier Phagocytosis

Several theoretical models have been proposed in order to understand and ultimately predict phagocytosis, depending on the nanoparticles characteristics. Based on the idea that nanoparticle phagocytosis is primarily an interaction between two surfaces, a first model has been anticipated using the so-called wettability hypothesis. According to this model, the probability of phagocytosis is related to the wettability (measured by the contact angle) of the cell membrane in comparison to that of the particle surface (Drummond and Fong 1999). In other words, this model is limited to the hydrophilicity/hydrophobicity characteristics of the nanoparticles. A more recent theory is based on the hypothesis that the film tension existing between the particle and the cell during the early and intermediate stages of phagocytosis plays a critical role in the mediation of the particle engulfment. This more comprehensive model allows to take additional forces into account, such the as electrostatic, van der Waals, receptor-ligand (FcR-Fc) and cytoskeletal (actin polymerization) forces, allowing refined predictions based on nanoparticle size and surface properties (Champion and Mitragotri 2006).

3.2 Other Endocytic Pathways

3.2.1 Experimental Techniques

In addition to the above-described methodologies to study the phagocytosis—most of which are also applicable to investigate various endocytosis pathways, specific markers (Table 1) can be tracked using various inhibitors to characterize CME [e.g, chlorpromazine, known to disrupt the assembly of clathrin-coated pits (Wang et al. 1993)], CvME [e.g, nystatin and filipin (Schnitzer et al. 1994)] and macropinocytosis [amilorid

Table 1 Major markers of subcellular compartments

Markers	Subcellular compartments
Caveolin	Caveolae
EAA1	Early endosomes
ESCRTs	Late endosomes
GM130 or giantin	Cis-Golgi or cis/mid-Golgi cisternae
LAMP-1	Lysosomes
LDL	Clathrin-coated pits
LysoTracker	Lysosomes and late endosomes (pH < 5.2)
Rab 7	Late endosome
Rab 11	Recycling compartment
TGN46	Trans-Golgi
Transferrin	Clathrin-coated pits; early and recycling endosomes

(Swanson 1989; Hoffmann et al. 2001)]. The specific inhibition of CME and CvME has also been recently achieved through infection with adenoviruses encoding mutant specific endocytic peptides, prior to incubation with nanoparticles (Harush-Frenkel et al. 2007). The characterization of RME is often performed using competition studies, while techniques like surface plasmon resonance allow to quantify the interaction between receptors and ligand-decorated nanoparticles (Stella et al. 2000). Atomic force microscopy was also recently used to quantify the interaction between a cell and nanoparticles deposited on the probe tip (Vasir and Labhasetwar 2008).

Finally, the uptake studies performed on cocultures of different cell lines require the identification of each cell type simultaneously to the detection of the nanocarriers, which can be achieved using internalized fluorescent dyes in confocal fluorescence microscopy, or using specific antibodies in flow cytometry (Grabowski et al. 2016) (Fig. 2).

3.2.2 Nanocarrier Characteristics Influencing Non-phagocytic Endocytosis

Contrary to phagocytosis, it is difficult to describe a thorough and consistent profile of nanoparticle matching each of the above-mentioned endocytic pathway. Indeed, unlike phagocytosis occurring primarily in professional phagocytes, other endocytic mechanisms may take place in virtually all types of cells and vary accordingly; differences may also occur between the apical and basolateral membranes of a polarized cell (von Bonsdorff et al. 1985). Moreover, several endocytic mechanisms often take place simultaneously.

Size

Nanoparticle size is a relevant parameter regarding the endocytic pathway, although its impact may vary upon the type of cells. For example, the same polystyrene nanoparticles (varying from around 20–1000 nm) were not preferably endocyted according to their size by the HUVEC endothelial, the ECV 304 bladder carcinoma and the HNX 14C squamous carinoma cell lines, whereas the 20–100 nm particles were preferentially internalized by the Hepa 1-6 hepatoma and the HepG2 human hepatocyte cell lines and the 20–600 nm particles by the KLN 205 squamous carcinoma cell line (Zauner et al. 2001). Some trends can however be drawn.

Cells from the gastrointestinal epithelium [Caco-2 cell line (Desai et al. 1997) as well as rat gastrointestinal tissue (Desai et al. 1996)] display a greater uptake for 100 nm PLGA particles compared to 0.5–10 μm ones, both in terms of number and total mass. The same size-dependency was observed on conjunctival epithelial cells in vivo for PLGA particles (Qaddoumi et al. 2004), as well as for poly(ε-caprolactone) (PCL) particles (Calvo et al. 1996). In the 1–100 nm range, explored more recently, the highest uptake (in number) was found with 50 nm gold beads (Devika Chithrani et al. 2006; Jiang et al. 2008) for Hela cells, occurring via receptor-mediated endocytosis.

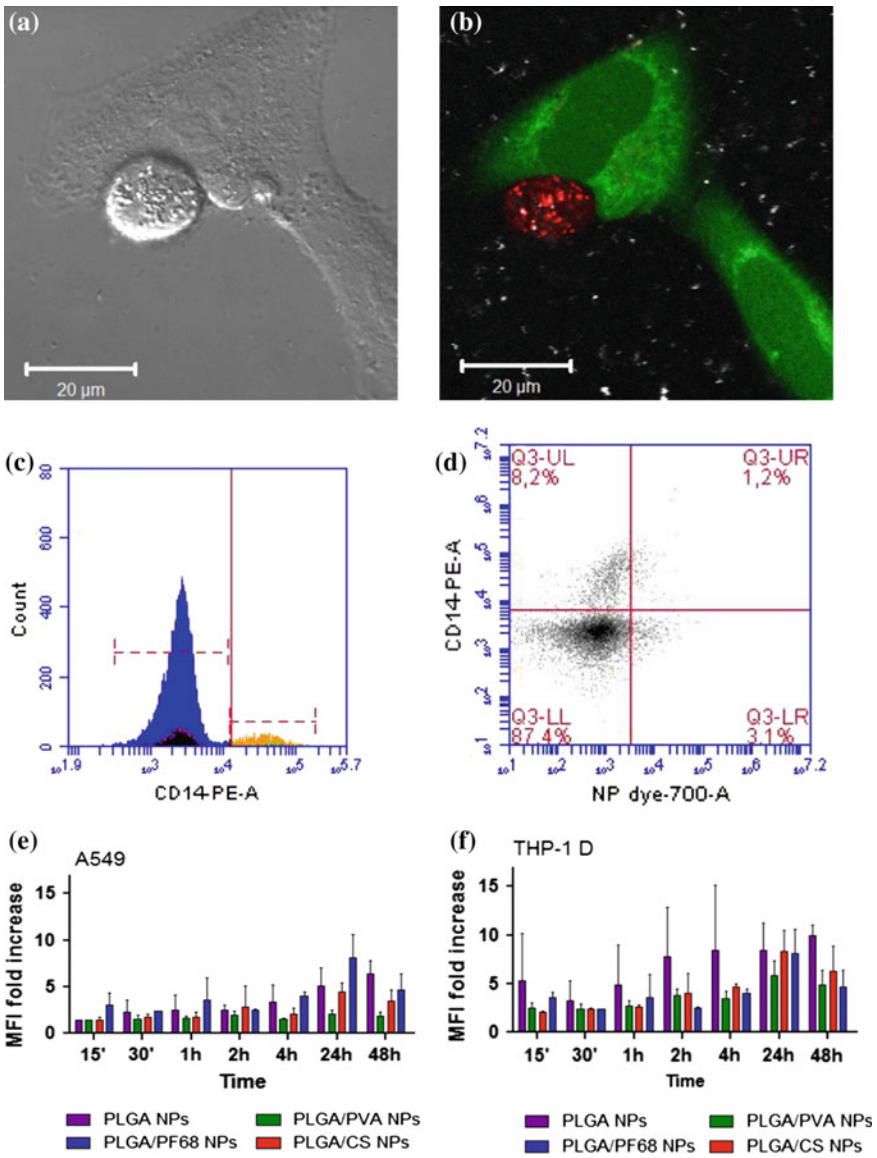

Fig. 2 Investigation of nanoparticle uptake kinetics by two cell subpopulations cocultured simultaneously. **a**, **b** Microscopic observations in phase contrast (**a**) and confocal fluorescence (**b**) of THP-1 macrophages (labeled by orange Lyzotracker, colored in *red*) in contact with A549 pulmonary epithelial cells (labeled by carboxyfluorescein diacetate succinimidyl ester, colored in *green*) exposed to PLGA nanoparticles (labeled by Dyomics DY-700, appear as *white dots*). All fluorescent probes have been selected to avoid emission spectrum overlap. **c**, **d** Flow cytometry quantification of nanoparticle uptake. Cell subpopulations are identified by fluorescent CD14 antibodies (**c**) simultaneously with particle uptake by DY-700 dye (**d**). **e**, **f** Nanoparticle uptake is represented as the increase of mean fluorescence intensity (MFI) compared to corresponding untreated cells, for both cell subpopulations (Grabowski et al. 2016)

The size may also directly affect the mode of endocytosis. Although the typical endosome sizes reported (i.e., 100 nm for CME, 50–80 nm for CvME) (Bareford and Swaan 2007; Conner and Schmid 2003; van Oss 1978; Chen et al. 1997) do not perfectly match the sizes of drug nanocarriers (most of the time higher than 100 nm) (Desai et al. 1996, 1997; Calvo et al. 1996), some size-dependant endocytosis pathways have been reported in the non-phagocytic murine melanoma B16 cells, using 50–1000 nm polystyrene beads devoid of ligands (Rejman et al. 2004). Internalization of nanoparticles having a diameter below 200 nm was found to involve CME. As the size of the particle increased, a shift to the CvME internalization pathway became apparent and turned to be the predominant pathway for particles as big as 500 nm. Thus, CME occurred for nanoparticles with a size limit of around 200 nm and kinetic parameters seemed to determine internalization of these particles along CME rather than CvME (Rejman et al. 2004). More studies are required to understand the CvME uptake of the biggest particles. On the other hand, looking at the lower end of nanocarrier size, alternative pathways to CME and CvME have recently been proposed. In particular, studies on polystyrene nanoparticles internalization by HeLa cells showed that, while beads of 40 nm in diameter entered cell through well-known CME, particles smaller than 25 nm were internalized via a novel non-clathrin and non-caveolae-mediated pathway, being also cholesterol-independent (Lai et al. 2007), which may open the door to the design of new drug delivery nanocarriers.

Finally, macropinocytosis corresponds to a poor size-selective endocytosis pathway, generally occurring in complement of CME or CvME (Harush-Frenkel et al. 2008; de Rieux et al. 2007). In some cases, size may however have little influence on uptake (Prabha et al. 2002), compared to surface properties (e.g. charge and presence of ligands).

Surface Charge

Due to the negatively charged character of the cell plasma membrane, positively charged drug nanocarriers generally display better association and internalization rates. Such nanoparticles are generally based on (or coated with) cationic polymers, the most widely used being the polysaccharide chitosan. Several studies report an efficient uptake by Caco-2 cells of cross-linked chitosan nanoparticles [e.g., particles having a zeta potential $\zeta \approx +15$ to $+30$ mV (Ma and Lim 2003; Mao et al. 2005)] through adsorptive endocytosis, and possibly involving CME. Similar patterns were observed on A-549 epithelial cells (Huang et al. 2002, 2004). Other cationic nanocarriers similarly impact endocytosis, such as PLA-PEG – based nanoparticles coated with the cationic lipid stearylamine ($\zeta \approx +35$ mV) which showed increased and faster uptake by HeLa cells compared to the negatively charged parent PLA-PEG nanoparticles ($\zeta \approx -35$ mV). The former was processed through the CME pathway, contrarily to the latter (Harush-Frenkel et al. 2007). Some authors, using quaternary ammoniums to modulate the surface charge of mesoporous silica nanoparticles ($\zeta \approx -5$ to $+20$ mV), suggested a threshold of

positive surface charges on endocytosis, depending also on the cell line used (Chung et al. 2007). Interestingly, a positive charge resulting from drug adsorption may also impact on the nanocarrier's endocytosis, as shown with PCL nanoparticles loaded with tamoxifen on MCF-7 breast cancer cells (Chawla and Amiji 2002); however, the drug leakage should be taken into account.

Nonionic Surface Coating

Coating nanoparticle by nonionic polymers like PEG can also influence endocytosis, as suggested by several studies focused on the interaction between various PEGylated nanoparticles and brain endothelial cells. It was shown that nanospheres prepared from a poly(methoxyethyleneglycol cyanoacrylate-co-n-hexadecyl cyanoacrylate) (PEG-PHDCA) copolymer were able to accumulate in both healthy rat brain and brain glioma (Calvo et al. 2001a, b, 2002; Brigger et al. 2002) not only owing to a prolonged blood circulation time, but also to a specific affinity of the surface of these nanoparticles for the endothelial cells of the blood–brain barrier (BBB) (Brigger et al. 2002). Using an original in vitro model of rat BBB (Garcia-Garcia et al. 2005), the authors shown that PEG-PHDCA were internalized through the CME pathway after specific recognition by LDL receptors, and accumulated in endosomal/lysosomal compartments (Kim et al. 2007). While the total amount of adsorbed proteins was lower onto the PEG-PHDCA nanoparticles than on their PHDCA counterparts, a preferential adsorption of apolipoprotein E (apo E) onto PEG-PHDCA nanoparticles was correlated with their increased cell uptake, thus suggesting the critical role of this protein in the endocytosis of these particles by the rat brain endothelial cells (Kim et al. 2007a, b). Similar conclusions were drawn from parallel studies performed on PACA nanoparticles PEGylated by the single adsorption of polysorbate 80. Such nanoparticles have indeed shown efficient endocytosis by brain endothelial cells using various labels (Kreuter 2001). Here also, a preferential adsorption of apo E (and/or apo B) on these particles suggested that the particles may undergo endocytosis after specific binding to LDL receptors (Kreuter 2001, 2002). Adsorption of polysorbate 80 onto solid lipid nanoparticles (SLN) and PLA nanoparticles has also been investigated along the same line (Göppert and Müller 2003, 2005; Sun et al. 2004). Although some controversy arose about possible interactions between the desorbed surfactant molecules and the cell tight junctions (Olivier et al. 1999; Kreuter et al. 2003), this was not the case with nanoparticles prepared from the PEG-PHDCA copolymer where the PEG chains are chemically linked, thus firmly bound at the surface of the particles.

Decoration by Targeting Ligands

The decoration of nanoparticles by targeting ligands, i.e, molecules able to recognize a specific biological target, has been investigated in order to promote delivery to a specific cell population and/or to control the intracellular trafficking of

the nanocarriers. This strategy relies on the idea that ligand-bearing nanocarriers will be internalized through the same pathway as the ligand alone. Moreover, the concentration of ligands on the nanoparticles surface offers potential for stronger cell interactions as compared to ligand alones. Indeed, the entropic gain in the formation of multivalent complexes may increase the binding constants by a factor of 1000 for bivalent interactions and even by 10^8 for tri- and pentavalent ones (Haag and Kratz 2006).

The folic acid (FA) vitamin has been widely studied as a targeting ligand for nanocarriers, especially for anticancer strategies (Chavanpatil et al. 2006). Indeed, FA binds with a low affinity to the reduced folate carrier present in virtually all cells, but with a high affinity (in the nanomolar range) to the glycosylphosphatidylinositol-linked folate receptor (FR), which exhibits highly limited distribution (Haag and Kratz 2006). In particular, FR is often overexpressed on the surface of cancer cells but highly restricted in normal tissues (Hilgenbrink and Low 2005). Moreover, FR has the ability to transport both FA and the FA-linked cargo by RME with subsequent endosomal escape into the cytosol (Haag and Kratz 2006; Weitman et al. 1992), thus avoiding lysosomal degradation. Although CvME appears to be involved in the uptake of FA in some cases (Rothberg et al. 1990; Pelkmans and Helenius 2002), the complete mechanism is complex and remains debated (Haag and Kratz 2006; Dauty et al. 2002). In the case of polymer nanocarriers, plasmon surface resonance revealed that FA covalently bound to PEG-PHDCA nanoparticles had a tenfold higher apparent affinity for FR compared to free FA (Stella et al. 2000). Liposomes were also decorated with FA by incorporating a phospholipid-anchored FA (Sabharanjak and Mayor 2004) or a FA-PEG-phospholipid conjugate (Lee and Low 1995) into the liposome bilayer. Such liposomes have shown a preferential uptake by FR-expressing cells. Similar cell uptake data were obtained with PLGA nanoparticles coated with the poly (L-lysine)-PEG-FA conjugate (Gabizon et al. 2004), with albumin nanoparticles coated with activated FA (Chavanpatil et al. 2006), as well as with polymer micelles prepared from a mixture of poly(L-histidine)-PEG-FA and PLA-PEG-FA (Kim et al. 2005).

Transferrin (Tf) has also been studied as targeting ligand to specific cell populations in order to increase cellular uptake of nanocarriers. Indeed Tf receptors (TfR) are overexpressed in several malignant tissues compared to healthy ones (Lee et al. 2005) [typically twofold to tenfold more (Qian et al. 2002)]. PLGA nanoparticles conjugated with Tf exhibited a twofold greater in vitro uptake by MCF-7 cells as well as a reduced exocytosis, compared to unconjugated PLGA particles; competition experiments with free Tf confirmed the involvement of TfR in the uptake process (Vasir and Labhasetwar 2007). In vivo studies performed in S-180 solid tumor-bearing mice showed a promising accumulation in the tumor of paclitaxel after intravenous administration of Tf-conjugated to PEG-PACA nanoparticles loaded with this drug (Sahoo and Labhasetwar 2005). TfR are also known to be highly expressed in some healthy tissues like brain capillaries where they are known to mediate transcytosis (Jones and Shusta 2007). Interestingly, Tf-conjugated to PEG-coated albumin nanoparticles significantly increased the

delivery of AZT to rat brain, the proportion of the drug located in this tissue being doubled as compared to the same nanoparticles devoid of ligand (Xu et al. 2005).

The use of ligands like Tf for nanocarrier functionalization may however be hindered by a competition with the corresponding endogenous pool of ligands (Jones and Shusta 2007). This is the reason why monoclonal antibodies (MAb) have been employed, as for instance the mouse OX26 directed against the rat TfR. This MAb binds to a TfR epitope dinstinct from the Tf binding site, thus preventing competition with endogenous Tf (Mishra et al. 2006). In this context, OX26 has been conjugated to PEGylated liposomes to increase the brain delivery of the encapsulated drug daunomycin to rats (Lee et al. 2000; Huwyler et al. 1997). The transcytosis mechanism of such OX26 immunoliposomes was demonstrated using an in vitro model of BBB consisting in a monolayer of rat brain endothelial cells RBE4 (Maruyama et al. 1995). Similar studies were carried out on mice, but using another MAb, the rat 8D3 MAb to the mouse TfR (Mishra et al. 2006). It was also observed that PEGylated immunoliposomes decorated with the Fab' fragments of antibodies reduced the RES uptake that is observed when using the whole antibodies (Cerletti et al. 2000) whose Fc fragment may be recognized by macrophages (Maruyama et al. 1997). Using the avidin (SA)-biotin (BIO) technology, chitosan nanospheres were also conjugated with PEG bearing the OX26 MAb. These functionalized chitosan-PEG-BIO-SA/OX26 nanoparticles were able to translocate into the brain tissue after intravenous administration (Harding et al. 1997). A high density of antibodies at the nanocarrier surface may, however, increase hydrophobicity, thus limiting the ability to escape the recognition by the RES. To combine efficient targeting and minimal RES uptake, an optimal coating of 10–30 antibody molecules per particle has been suggested in the case of liposomes (Aktaş et al. 2005; Andresen et al. 2005). For example, a density of around 30 OX26 antibody molecules per (100 nm) liposome was found optimal for brain delivery to rats in two independent studies (Huwyler et al. 1997; Maruyama et al. 1999).

Lectins have also attracted interest because of their inherent ability to provide specific binding to carbohydrates located at the surface of epithelial cells (Huwyler et al. 1996). Tomato lectins (TL) have been intensively utilized for the delivery to the intestinal mucosa after oral administration, as TL-coated particles were shown to avidly adhere to enterocytes both in vitro (Ponchel and Irache 1998) and in vivo (Lehr et al. 1992). Other lectins like wheat germ agglutinin (WGA) have the ability to bind cancer cells preferentially to normal cells (Florence et al. 1995). Interestingly, PLGA nanoparticles coated with WGA exhibited a preferential uptake by A549 and H1299 cancer cells (Aub et al. 1963; Mo and Lim 2005).

Ligands of cell adhesion molecules (CAMs) have been more recently investigated for the targeting of various endothelial cells. In particular, RGD peptides have been used to target tumor cells with increased expression of specific CAM integrins (Mo and Lim 2005). For example, PEGylated liposomes conjugated with the RGD peptide were found to form clusters on endothelial microvessels of tumors in mice, contrary to control liposomes conjugated with a RAD peptide (Dunehoo et al. 2006). 'Intracellular' CAM-1 (ICAM-1) is another particularly interesting target for

perturbed endothelial cells (Schiffelers et al. 2003). Polystyrene nanoparticles bearing MAb to human and mouse ICAM-1 were developed for this purpose (Ding et al. 2006). Interestingly, endothelial cells did not internalize ICAM-1 MAb but well MAb-coated nanoparticles or multivalent MAb conjugates. Indeed, the uptake was found to require ICAM-1 clustering. The endocytosis pathway was independent from CME, CvME, macropinocytosis and phagocytosis (Muro et al. 2003, 2006). The nanoparticles finally trafficked to lysosomes (Muro et al. 2003). In vivo studies showed that nanoparticles conjugated with ICAM-1 MAb enabled also vascular delivery to pulmonary and vascular endothelium (Muro et al. 2006).

Shape

The influence of particle shape on endocytosis is being increasingly investigated. In some cases, it was found that spherical nanoparticles had a higher and faster rate of endocytosis compared to rods or disks, as demonstrated using gold nanoparticles (Devika Chithrani et al. 2006) as well as ICAM-1- and TAT-coated nanoparticles (Christofidou-Solomidou et al. 2000; Muro et al. 2008). On the contrary, other studies suggested preferential uptake of rod-shaped (Zhang et al. 2008) or cylindrical (Gratton et al. 2008) particles.

3.2.3 Theoretical Models

Several models have been proposed to describe the non-phagocytic endocytosis of viruses and nanoparticles. A first model of RME based on the hypothesis of a cell membrane containing diffusive mobile receptors, wrapping around a particle coated with corresponding ligands, leads to a threshold particle radius of around 30 nm, above which endocytosis is likely to occur (Gratton et al. 2008). Then, a more general mechanism has been proposed, which includes also nonspecific attractive/repulsive forces. This contribution, which was found to be as important as specific interactions, leads to more general predictions (Gao et al. 2005). The importance of elastic deformation of the cytoskeleton for the engulfment has also been shown to be of significant contribution (Decuzzi and Ferrari 2007). However, the variety of the mechanisms of non-phagocytic internalization pathways seems to complicate the elaboration of a general model, able to fit the multiple experimental data.

4 Investigation of Nanoparticle Intracellular Trafficking

In addition to above-mentioned techniques, the intracellular trafficking can be monitored using fluorescent markers specific for vesicles (e.g, Lysotracker® for the endo/lysosomes). Cell fractionation techniques are also used, such as a selective permeation of membranes, in which sequential treatments with proteases and

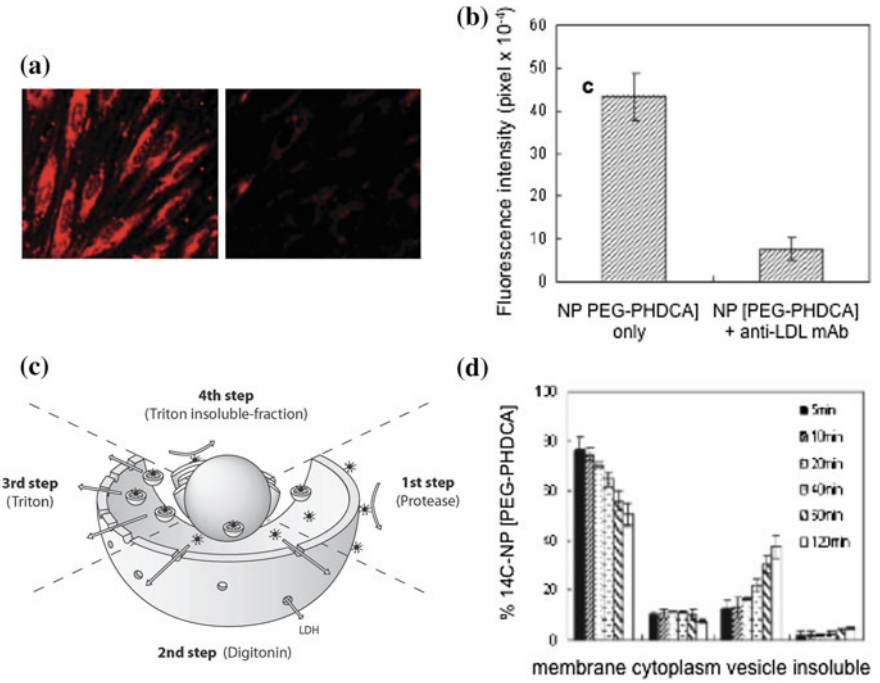

Fig. 3 Investigation of nanoparticle subcellular distribution following uptake by endothelial cells. **a**, **b** Semi-quantification of internalized PEG-PHDCA nanoparticles labeled by Nile *red* in primary rat endothelial cells from the blood brain barrier, showing the involvement of LDL receptors. **c** Cell fractionation methodology, based on selective membrane permeation or desorption, allowing access to cytoplasmic membrane-bound, cytosolic, endo/lysosome and residual fractions (Eboue et al. 2003). **d** Quantification using ^{14}C-labeled PLGA over 2 h exposure, showing a progressive endosomal sequestration. Reproduced from Kim et al. (2007); Garcia-Garcia et al. (2005b). Source: Elsevier

surfactants allow the recovery and the quantification of (fluorescence or radiolabeled) material associated to the plasmic membrane, cytosol, endo/lysosomal vesicles and other organelles (Sun and Wirtz 2006) (Fig. 3).

With respect to nanocarrier characteristics, the particle electric charge plays a crucial role in its intracellular fate. This is of particular importance in its interaction with endocytic vesicles, in response to the pH decrease during endosome maturation and fusion with lysosomes. Most strategies developed in this field aim at promoting endosomal escape in order to limit drug degradation due to low pH and presence of enzymes, and to ensure the cytosolic delivery of the drug when needed.

pH-sensitive liposomes have been tailored for this purpose. Most of them are based on dioleyl phosphatidylethanolamine (DOPE), which undergoes a transition from lamellar to inverted micelles structures at low pH, allowing the fusion between the liposomal and the endosomal membranes, and the destabilization of the endosomes (Garcia-Garcia et al. 2005; Fattal et al. 2004). DOPE is often used in combination with the mildly acidic amphiphils oleic acid (OA) and cholesteryl

hemisuccinate (CHEMS). At neutral pH, OA and CHEMS (ionized) act as stabilizers and allow DOPE to maintain a bilayer structure; at lower pH, OA and CHEMS get protonation and cause the destabilization of the liposomal bilayer with the subsequent release of the liposome content (Zuhorn et al. 2007). Typically, DOPE:OA liposomes become leaky at pH 6.5 and DOPE:CHEMS at pH 5.5 (Ellens et al. 1984). The transfer of the DOPE molecules to the endosomal membrane is thought to promote endosome leakage (Garcia-Garcia et al. 2005; Drummond et al. 2000), although the precise mechanism remains to be elucidated (Qian et al. 2002). Such pH-sensitive liposomes have shown efficient in vitro cytosolic delivery of model fluorescent probes (Bergstrand et al. 2003) and oligonucleotides (ODN) (Straubinger et al. 1985; Ropert et al. 1992). However, the in vivo efficacy is more questionable, mainly due to stability concerns in the presence of serum (Düzgünes et al. 2001).

Unlike these pH-sensitive anionic liposomes, lipoplexes, resulting from the complexation of nucleic acids with cationic lipids, exhibit a total net positive charge (Qian et al. 2002; Fattal et al. 2004). They are often designed using the cationic DOTMA (Connor et al. 1986) and DOTAP (Felgner et al. 1994). Although they display some in vitro transfection activity, their efficacy runs short from a therapeutic point of view. This was attributed to an inefficient destabilization of the endosomal membranes (Fattal et al. 2004).

On the contrary, if cationic polymers do not possess fusogenic activity per se, some of them, like the popular poly(ethylene imine) (PEI), have however the ability to disrupt endosomal membrane, as a consequence of their important buffering capacity (Simberg et al. 2004). Indeed, polyplexes resulting from the complexation of DNA plasmids with PEI show remarkable transfection efficiency on various cell lines, which lead the authors to propose the so-called ‘proton-sponge’ effect (Demeneix et al. 2004). According to this hypothesis, the endosomal pH decrease entails a high protonation of PEI, which results in an osmotic swelling due to water entry and subsequent vacuole disruption, thus allowing the cytoplasmic release of the PEI/DNA particles (Demeneix et al. 2004). This was supported by the fact that PEI transfection efficiency was decreased by 100-fold by bafilomycin-A, a specific vacuolar H^+-ATPase inhibitor (Boussif et al. 1995). Noteworthy, live cell microscopy also allowed to visualize a burst release of polyplexes from the acidic vesicles (Kichler et al. 2001). Other mechanisms have also been suggested, such as a possible swelling of the polymer network resulting from the increasing repulsion of the protonated groups (Merdan et al. 2002). Based on electron microscopy observations, direct interactions with the lysosomal membrane leading to the formation of holes have been proposed too (Behr 1997). The precise mechanism of the endo/lysosomal escape as well as the transport to the nucleus is not yet clearly understood. However, PEI remains today one of the major transfection agents useful for the design of nanocarriers able to escape the endosomes. Recent studies aim at clarifying the impact of the physicochemical characteristics of PEI (structure, molecular weight) and its polyplexes on the transfection efficiency (Bieber et al. 2002).

Combining such features promoting endosomal escape with stealth properties in the same nanocarriers is often challenging. Some developments of nanocarriers

based on modified PEG have paved the way for new pH-responsive systems, the key feature being the incorporation of acid-labile groups. For instance, polymer micelles loaded with the anticancer drug doxorubicin were prepared from PEG-dendrimer hybrids on which hydrophobic groups were attached through an acid sensitive acetal linkage (Neu et al. 2005). The micelles were stable at pH 7.4, but upon acidification of endosomes, the loss of hydrophobic groups by hydrolysis caused the destabilization of the micelles, which enabled drug release (Gillies et al. 2004). Another recent example involves the self-assembling of the amphiphilic block copolymers PEG-poly(aspartate), to which the anticancer drug adriamycin was conjugated through hydrazone linkers that were stable at pH 7, but cleavable at pH 6 and below. The micelles formed by this copolymer were taken up in vitro by the cells of a multicellular tumor spheroid and the released drug was observed to accumulate in the cell nuclei, suggesting that escape from endo/lysosomes has taken place (Gillies and Fréchet 2005).

The decoration of nanocarrier surface with ligands also determines its intracellular fate. Cell penetrating peptides (CPPs), also known as protein transduction domains, have also raised increasing attention due to their ability to translocate across membranes (Bae et al. 2005; Patel et al. 2007). The most commonly studied CPP for nanoparticle functionalization is the HIV-1 trans-activating transcriptional activator peptide (TAT). Remarkably, ultrasmall superparamagnetic iron oxide particles (USPIO) coated with TAT were shown to efficiently tag progenitor cells (Torchilin 2008). An increasing number of examples of conjugation of TAT to liposomes (Lewin et al. 2000), polymer micelles and polyplexes (Yagi et al. 2007) have been described. However, the internalization mechanism of CPPs remains to be fully elucidated: it may involve macropinocytosis (Bae et al. 2005), but also CME and CvME (Patel et al. 2007), as well as direct penetration, although the latter remains debated. Endosomal escape of CPPs and nuclear targeting also need further investigations.

Other ligands have been recently investigated to address nanocarriers to intracellular organelles, like mitochondria and nucleus. The few examples of mitochondrial targeting of nanoparticles include the binding of the peptide sequence Mito-8 to quantum dots (QDs), which showed strong in vitro mitochondrial localization (Kleemann et al. 2005). Nucleus targeting of nanoparticles is actively investigated for gene delivery. Promising experiments have been carried out using nuclear localization signals (NLS) peptides (Hoshino et al. 2004) or TAT peptides (Tkachenko et al. 2003) coated onto gold nanoparticles.

Finally, the influence of particle shape on the intracellular trafficking also deserves more insight. A recent study has compared layered double hydroxides (LDHs) nanoparticles made of Mg and Al oxides, having hexagonal or rod shapes (de la Fuente and Berry 2005). Both were internalized by various mammalian cell lines through CME and were found to escape from endosomes (probably through their buffering capacity), but hexagonal LDHs remained in the cytoplasm whereas rod-like LDHs were directed to the nucleus, probably through a microtubule-mediated active transport mechanism (de la Fuente and Berry 2005). This opens exciting perspectives, especially for the control of the intracellular gene delivery.

5 Concluding Remarks

While significant progress in the understanding of the nanoparticle internalization by a variety of mammalian cells has already been achieved, many advances in nanomedicine still rely on phagocytosis, while tailoring nanocarriers targeting the other endocytic pathways is complicated by the variety of the cells and internalization mechanisms encountered. Modeling such complex biological barriers with reliable in vitro systems remains a difficulty, together with the disparities in the experimental conditions used to study the nanoparticle-cell interactions. Despite these hurdles, the expending knowledge of biological markers offers increasing possibilities to target nanocarriers to and inside the desired cell populations.

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