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REVIEW

Nanocarriers' entry into the cell: relevance to drug delivery

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Abstract Nanocarriers offer unique possibilities to overcome cellular barriers in order to improve the delivery of various drugs and drug candidates, including the promising therapeutic biomacromolecules (i.e., nucleic acids, proteins). There are various mechanisms of nanocarrier cell internalization that are dramatically influenced by nanoparticles' physicochemical properties. Depending on the cellular uptake and intracellular trafficking, different pharmacological applications may be considered. This review will discuss these opportunities, starting with the phagocytosis pathway, which, being increasingly well characterized and understood, has allowed several successes in the treatment of certain cancers and infectious diseases. On the other hand, the non-phagocytic pathways encompass various complicated mechanisms, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis, which are more challenging to control for pharmaceutical drug delivery applications. Nevertheless, various strategies are being actively investigated in order to tailor nanocarriers able to deliver anticancer agents, nucleic acids, proteins and peptides for therapeutic applications by these non-phagocytic routes.

Keywords Liposomes · Nanoparticles · Polymeric micelles · Endocytosis · Phagocytosis · Clathrin · Caveolae · Macropinocytosis

Abbreviations

BBB	Blood-brain barrier
CME	Clathrin-mediated endocytosis
CvME	Caveolae-mediated endocytosis
FA	Folic acid
HSA	Human serum albumin
ICAM-1	Intracellular cell adhesion molecule 1
MAb	Monoclonal antibody
ODN	Oligonucleotide
PACA	Poly(alkylcyanoacrylate)
PEG	Poly(ethyleneglycol)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-*co*-glycolic acid)
PSt	Polystyrene
RES	Reticuloendothelial system
RME	Receptor-mediated endocytosis
siRNA	Short-interfering RNA
TAT	Trans-activating transcriptional activator peptide
Tf	Transferrin
TfR	Transferrin receptor

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Introduction

Drugs are acting through many mechanisms. In some of them, passing through the cell membrane in a cell-type or tissue-specific manner is needed, especially when the relevant pharmacological target is located intracellularly. In this case, a complex series of interactions with the cells of

the body is required. Indeed, the therapeutic molecule must generally: (1) cross one or various biological membranes (e.g., mucosa, epithelium, endothelium) before (2) diffusing through the plasma membrane to (3) finally gain access to the appropriate organelle where the biological target is located. For those drugs whose target is located intracellularly, deviating from this ideal path may not only 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 1970s, the proof of concept was done showing that submicronic lipid vesicles known as liposomes [1], as well as synthetic polymer nanoparticles [2], 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. This approach has been exploited to optimize the intracellular delivery of many small molecules as well as of macromolecules like nucleic acids, peptides or proteins, which are unstable in physiological conditions and generally unable to cross the cell membrane.

This review describes the cell capture pathways as well as the intracellular trafficking of nanodevices useful for drug delivery (vaccines have been reviewed elsewhere [3]). The influence of the nanocarriers' physico-chemical properties on their interaction with cells is also discussed, in order to design the more efficient drug targeting strategies. We will focus on the main particulate submicronic systems developed to date in the field: liposomes, and polymer-based nanoparticles or micelles. Liposomes are lipidic vesicles, formed by one or several phospholipids bilayers surrounding an aqueous core (Fig. 1a). Polymeric nanoparticles are generally based either on synthetic biodegradable polymers—like the poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) polyesters or the poly(alkylcyanoacrylates) (PACA)—or natural polymers, like albumin. Nanospheres are matrix systems in which the drug is dispersed throughout the particles (Fig. 1b), whereas nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymeric membrane (Fig. 1c). Other systems based on polymers include polymer micelles (Fig. 1d), dendritic architectures and polyplexes (formed by the electrostatic interactions of polycations and nucleic acids). To avoid redundancy, the cell interaction of dendrimers will be discussed only when they display unique or specific properties.

According to the physicochemical characteristics of the nanocarrier and the nature of the target cells, two main internalization pathways may occur: either the phagocytosis (Fig. 2a) or the other endocytic pathways (i.e., clathrin- and calveolae-mediated endocytosis) (Fig. 2b–e). Notably, depending on the drug physico-chemical characteristics, the internalization pathway, as well as the intracellular fate of the nanocarrier, is a key issue for the drug to be efficient. The release of the drug into the enzymatic environment of the lysosomes or directly in the cell cytoplasm will, indeed, have important impact on the pharmacological activity. This is a reason why the

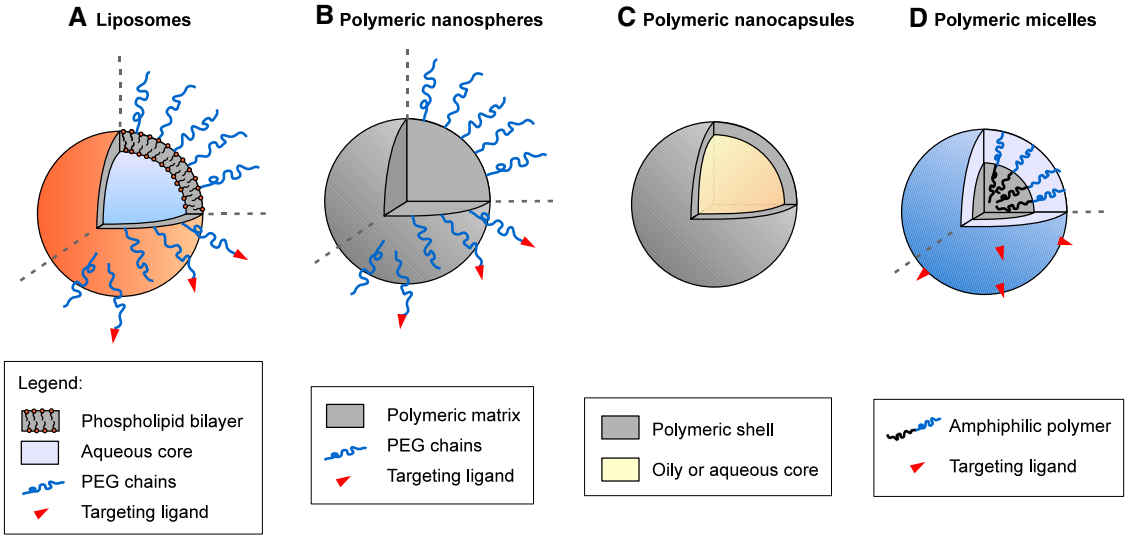

Fig. 1 Principal types of nanocarriers for drug delivery. **a** Liposomes are formed by one (or several) phospholipid bilayers surrounding an aqueous core. They can be PEGylated and decorated with targeting ligands. **b** Polymeric nanospheres are designed using biodegradable polyesters or poly(alkylacyanoacrylate), or natural polymers, like albumin. They can also be PEGylated and decorated with targeting ligands. **c** Polymeric nanocapsules are formed by a polymer membrane (same materials as for nanospheres) surrounding either an oily or an aqueous core. **d** Polymeric micelles are formed by the assembly of amphiphilic polymers, generally exhibiting a PEG shell that can be functionalized by targeting ligands

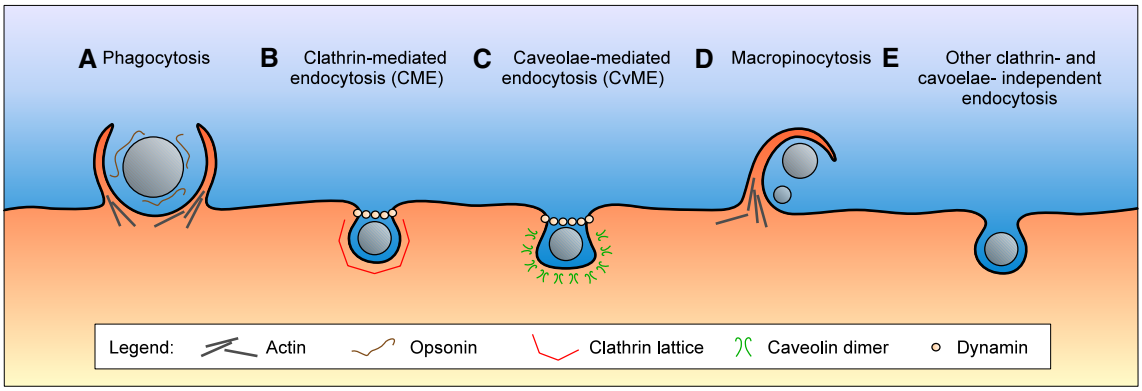

Fig. 2 Principal nanocarrier internalization pathways in mammalian cells. **a** Phagocytosis is an actin-based mechanism occurring primarily in professional phagocytes, such as macrophages, and closely associated with opsonization. **b** Clathrin-mediated endocytosis is a widely shared pathway of nanoparticle internalization, associated with the formation of a clathrin lattice and depending on the GTPase dynamin. **c** Caveolae-mediated endocytosis occurs in typical flask-shaped invaginations of the membrane coated with caveolin dimers, also depending on dynamin. **d** Macropinocytosis is an actin-based pathway, engulfing nanoparticles and the extracellular milieu with a poor selectivity. **e** Other endocytosis pathways can be involved in the nanoparticle internalization, independent of both clathrin and caveolae

present comprehensive review on the nanocarriers' entry into the cell may help to clarify the therapeutic benefit resulting from the use of nanodevices for the intracellular delivery of medicines.

Phagocytosis pathway

Phagocytosis plays a critical physiological role in the defense of the organism against nonself elements, infectious agents (most bacteria and some viruses) as well as exogenous inert particles—including drug delivery nanoparticles.

Mechanism of opsonization and phagocytosis

Phagocytosis occurs primarily in specialized cells, also called professional phagocytes: macrophages, monocytes, neutrophils and dendritic cell [4]. Other types of cells (fibroblasts, epithelial and endothelial cells), referred to as para- and nonprofessional phagocytes, may display some phagocytic activity, but to a lower extent [5]. The phagocytic pathway of entry into cells can be described using three distinct steps: recognition by opsonization in the bloodstream; adhesion of the opsonized particles to the macrophages; ingestion of the particle.

Opsonization is an important process occurring before the phagocytosis itself (Fig. 3a). 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. Major opsonins include immunoglubulins (Ig) G (and M) as well as complement components (C3, C4, C5) [6], in addition to other blood serum proteins (including laminin, fibronectin, C-reactive protein, type-I collagen) [7].

Opsonized particles then attach to the macrophage surface through specific receptor-ligand interactions (Fig. 3b). 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, the best understood interaction involving IgG and FcγR; CRs mostly bind to C3 fragments [4, 8]. Other receptors, including the mannose/fructose and scavenger receptors, can be involved in the phagocytosis [4], while new opsono-receptors like CD44 are still being discovered [9]. Receptor ligation is the beginning of a signaling cascade mediated by Rho-family GTPases [10], which triggers actin assembly, forming cell-surface extensions (pseudopodia) that zipper up around the particle and engulf it.

The resulting phagosome will ferry the particle throughout the cytoplasm (Fig. 3c). As actin is depolymerized from the phagosome, the newly denuded vacuole membrane becomes accessible to early endosomes [11]. Through a series of fusion and fission events, the vacuolar membrane and its contents will mature, fusing with late endosomes and ultimately lysosomes to form a phagolysosome (Fig. 3d). The rate of these events depends on the surface properties of the ingested particle, typically from half to several hours [4]. The phagolysosomes become acidified due to the vacuolar proton pump ATPase located in the membrane and acquire many enzymes, including esterases and cathepsins [12]. The enzymatic content of these intracellular vesicles is a key issue for synthetic polymeric nanoparticles, since polymer biodegradability is required in pharmaceutical applications, both to ensure drug release and to avoid accumulation of the ingested

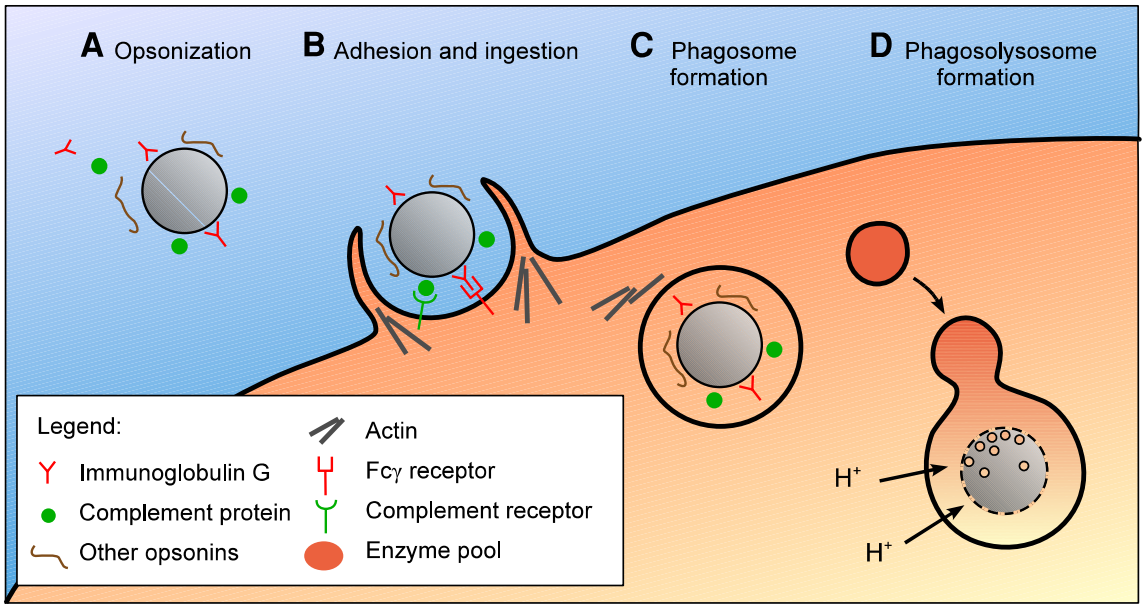

Fig. 3 Nanocarrier internalization by opsonization and phagocytosis. **a** Unless specifically designed, nanocarriers generally undergo extensive opsonization in the bloodstream, i.e., adsorption of immunoglobulins (mainly IgG), complement components (mainly C3) and other proteins like fibronectin. **b** The opsonized nanoparticles bind to the cell surface through specific recognition of the opsonins, trigerring actin assembly and particle engulfment. **c** The resulting phagosome matures, fuses with lysosomes and becomes acidified, leading to the enzyme-rich phagolysosomes (**d**) prone to particle degradation

material, which can lead to further toxicities. This explains the wide use of the biodegradable PLGA and PACA. PLGA chains are degraded through a hydrolytic mechanism facilitated by low pH [13], whereas PACA is bioeroded intracellularly, i.e., the alkyl groups are hydrolyzed by esterases, which increases the hydrophilicity of the polymer backbone until it becomes water soluble [14].

Nanocarrier characteristics influencing phagocytosis

Early studies performed on nanocarriers showed that liposomes [15] as well as polymeric nanoparticles [16] are rapidly cleared from the bloodstream by macrophages of the reticuloendothelial system (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. Several studies allowed to refine the profile of nanoparticles prone to phagocytosis and macrophage targeting.

Size

Although a minimum size of 0.5 μm for a particle to undergo phagocytosis is often put forward [4, 8], this statement is seldom [17] justified and generally used to highlight the wide size tolerance for phagocytosis—"macrophages can eat bigger than their head" [18]—compared to other endocytosis modes (see Sect. 2.). Model polystyrene (PSt) 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) [19]. Similarly, particles based on other polymers (HSA [20], modified cellulose [21], poly(methylmetacrylate) (PMMA) and PACA [20]) exhibited 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 [20]. Liposomes generally display the same pattern: larger (and multilamelar) ones (>100 nm) are less numerous, but deliver a higher payload to macrophages compared to the smaller (and unilamelar) ones [22]. On the other hand, other studies found a more balanced [23] or even opposite [24] 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) [25]. 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 [6].

Surface properties

The particle composition plays a critical role in phagocytosis in that it determines the physicochemical characteristics of the particle surface; thus, its ability to interact not only with the macrophage membrane, but also with opsonins prior to phagocytosis.

Interaction with opsonins In the presence of serum, the nature of the nanoparticle surface will directly influence the adsorption of opsonins (mainly complement proteins and Ig), and, in turn, the extent of phagocytosis. In general, 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) [26]. This trend translates well to the opsonization of nanocarriers. Indeed, highly charged particles have proven to fix complement proteins, especially liposomes [27], either negatively or positively charged, whatever the complement activation pathway [28]. 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 [29] (dysopsonins decrease recognition by phagocytes). More specifically, apolipoproteins have been proposed to contribute specifically to the uptake by hepatocytes [30]. In the case of polymeric nanoparticles, although negative charge can be related to a higher uptake [6, 31], the surface hydrophobicity appears to be the key factor for opsonization. Nanoparticles prepared from hydrophobic polymers PSt [32], PLA [33], PLGA [34] and PACA [35] 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 [36], as well as high affinity of IgG and albumin for hydrophobic regions [28] (van der Waals interactions may be a more accurate description for these "hydrophobic" interactions [37]).

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 polymeric 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(ethyleneglycol) [PEG, also known as poly(oxoethylene)] has been extensively described for this purpose [6, 7]. Liposomes as well as solid lipid nanoparticles can be PEGylated by anchoring PEG-phospholipids or PEG-lipids. PEGylated polymeric nanoparticles can be prepared either by adsorption of surfactants like poloxamers or polysorbates on already prepared nanoparticles, or by using preformed copolymers composed of PEG and a biodegradable moiety (polyesters [38] or PACA [39] mainly). PEG-based copolymers were also used for the PEGylation of polymeric micelles [40]. In general, these PEGylated nanodevices 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 a few minutes to several hours [41]. The stealthiness towards macrophages of these nanocarriers can be modulated by the length and density of the PEG chains on the nanoparticle surface, which impose the spatial conformation of the PEG moieties. More recently, polysaccharides have been studied as alternative hydrophilic polymers [42].

Interaction with the cell membrane It is noteworthy that polymeric 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 (PS) and phosphatidylglycerol (PG), have a much higher binding to and phagocytosis by macrophages as compared to neutral vesicles [23, 43]; the same is true for positively charged liposomes or polyplexes [44]. A similar pattern was found for negatively and positively charged polymeric nanoparticles compared to neutral ones [21, 31]. Additionally, hydrophobic nanoparticles are more readily captured than hydrophilic non-ionic ones [21] (in accordance with the "wettabily" theoretical model). 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 [45] and involvement of non-specific interactions with non-specific receptors by electrostatic interactions for negative particles [44], especially with type B scavenger receptor [46]. Taking advantage of macrophage receptors to enhance phagocytosis has been further achieved by coupling specific ligands to nanocarriers. For example, grafting rabbit Ig [47] as well as mouse monoclonal antibody [48] 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 the liposome surface, enhancing uptake by murine Kupffer cells and peritoneal macrophages [49]. New ways are still being explored, as attested by a study on the plasma membrane glycoprotein CD14 and the possible involvement of scavenger receptors [50].

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) [51]. Such macrophage's increased sensitivity to bead stiffness can be connected to the fact that bacteria and other pathogens have cell walls usually more rigid than the surrounding tissue they invade. 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 [24]. 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 [52]: a flexible particle is thought to provide a greater number of surface interactions with the biological environment. Thus, no clear relationship emerges between nanocarrier rigidity and phagocytosis.

Besides particle rigidity, recent works have focused on the control of the shape itself—making it different from spherical. Lipidic disks have been developed as alternatives to liposomes, having a diameter of 120 nm [53] to 250 nm [54], and a thickness of only a few nanometers, showing efficient uptake by RES macrophages. Other lipidic assemblies such as cube-shaped so-called cubosomes [55] have been recently 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 for polymeric systems, a recent study performed with PSt particles of various shape (ellipsoids, disks, UFO-like) has shown that, independently of opsonization, the local particle shape at the point of contact dictates whether macrophages initiate phagocytosis or simply spread on particles [56]. For example, a macrophage attached to an ellipse at the pointed end will internalize it in a few minutes, while a macrophage attached 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, even prevailed on particle size [56]. Control of the shape seems an appealing new way to control nanoparticle phagocytosis.

Drug delivery applications

The use of nanotechnologies to target RES organs has proven relevant in two major therapeutic areas: first, oncology, for the treatment of tumors, which although not being stricto sensu from the RES, are located in an organ of the RES (e.g., hepatocarcinoma and liver metastasis); second, infectiology, as many of pathogens that need to be eliminated (parasites, bacteria, viruses) are located in the RES macrophages.

Delivery of cytotoxic agents

One of the major applications that takes advantage of the preferential location of nanoparticles in the RES macrophages following intravenous administration is the treatment of hepatic tumors using PACA nanospheres. Kupffer cells are indeed major sites of accumulation of such particles (much more than endothelial and parenchymal cells) [57]. Thus, in a murine histiocytosarcoma hepatic metastases model [58], the cytotoxic doxorubicin loaded onto 200–300 nm PACA nanoparticles has proven superior to the free drug in terms of efficacy, reduction of cardiac toxicity and reversion of resistance [59]. Kupffer cells were found to act as a reservoir allowing slow diffusion of doxorubicin towards tumor cells, as a result of particle biodegradation [60]. However, phagocytosis was not the main mechanism of interaction between nanoparticle and cancer cells, as the in vitro drug uptake was not modified in the presence of cytochalasin B [61]. Instead, it was proposed that the formation of an ion pair between the positively charged doxorubicin and the negatively charged polycyanoacrylic acid (a by-product of PACA degradation) increased the diffusion of doxorubicin through the membrane [62]. This nanomedicine was first tested for tolerance in a phase I clinical trial [63] and has currently reached phase II/III under the name of Transdrug®.

Liposomes have also been developed along this line [64]. Conventional liposomes comprised of phosphatidylglycerol, phosphatidylcholine and cholesterol, in a range of 300–500 nm and loaded with doxorubicin, have shown a similar increased liver uptake and reduced cardiac toxicities in a phase I clinical study [65]. Here it was also suggested that after capture by Kupffer cells, the liposome matrix became leaky, and the drug was released in free form to the tumor [65].

Delivery of antiparasite and antifungus agents

Many efforts have been concentrated in using nanotechnologies to improve the delivery of amphotericin B, which is the leading compound against leishmaniasis and fungus infections. The goal has been to increase amphotericin B concentrations in RES tissues (e.g., the liver and the spleen) and to lower kidneys and lung concentration in an effort to reduce toxicity and to allow increased dosages. These strategies have been successful, as three new formulations are marketed today besides the classical one, Fungizone® (a micellar dispersion) [66]. Association of amphotericin B to 60–80 nm SUV liposomes has allowed a 50- to 70-fold decrease in toxicity and a subsequent 5-fold increase in the dose, leading to the approval of this formulation as AmBisome®. Notably, the small size of these liposomes also allows them to escape immediate clearance by macrophages of the liver and the spleen and thus to reach other infected tissues. [67]. Abelcet® was introduced as a cheaper formulation: the ribbon-like lipidic structure is bigger and show a higher RES uptake [66]. The already mentioned nanodisk formulations [53, 54] containing amphotericin B have also been developed as alternatives to AmBisome®, the 120-nm disk formulation being approved as Amphotec® [53].

Polymeric nanoparticles have also been investigated as antiparasite and antifugus delivery systems. For example, poly(isohexylcyanoacrylate) (PIHCA) nanospheres loaded with primaquinine increased by 21-fold the activity of the drug against intracellular *Leishmania* species [68]. In another study, PLA nanospheres promoted the activity and reduced toxicity of an experimental drug against leishmaniasis [69]. But on the whole, polymer-based nanocarriers have not reached the same stage of development as their lipid-based counterparts. Indeed, the biggest challenge is believed to be a higher cost.

Delivery of antibacterial agents

Nanotechnologies have also been used as a Trojan horse for the specific delivery of antibacterial agents into the infected macrophages. Indeed, many microorganisms are able to survive in phagocytes, where they may be protected from the host defense systems and from some antibiotics having a poor penetration in these cells. Infections by *M. tuberculosis* and *M. avium—M. intracellulare* (MAC) have received special attention because of rising incidence, often associated with AIDS. The encapsulation of various antibiotics in liposomes has been performed successfully, often showing a good antibacterial efficacy, both in vitro on macrophages cell lines and in vivo on animal MAC models [70]. In particular, a liposomal formulation of the aminoglycoside amikacin has shown a significant drug accumulation in tissues like liver and spleen compared to the free drug in a MAC murine model [71]. However, clinical trials were disappointing [72]. As for polymeric nanoparticles, poy(isobutylcyanoacrylate) (PIBCA) nanoparticles loaded with ampicillin significantly increased efficacy of the free drug in murine models of *Salmonella typhimurium* [73] and *Listeria monocytogenes* [74]. However, complete sterilization of the infected organs or elimination of the infection reservoir often proved to be difficult. These examples show the extreme difficulty to eradicate bacteria, even when a priori located in the RES, as well as the complexity to transpose in vitro results to in vivo human situations [41].

Delivery of antiviral agents

Macrophage targeting using polymeric nanoparticles has also been important for the delivery of antiviral drugs, especially anti-HIV, since macrophagic cells are known to be a reservoir for virus particles, also helping dissemination [75]. Thus, PACA nanospheres loaded with the reverse transcriptase inhibitor azidothymidine (AZT) have shown a preferential in vitro uptake by macrophages, especially the infected ones [20]. In vivo, these nanocarriers were able to efficiently concentrate AZT into the macrophages of the RES after intravenous administration [76], as well as in the gut-associated lymphoid macrophages after oral administration to rats [77]. Recent studies showed that PACA aqueous-core nanocapsules were even able to deliver in vitro to macrophages the active triphosphate form of AZT (which is too hydrophilic to cross the cell membrane) [78], opening perspectives to improve the potency of this class of antivirals and to overcome resistances. However, the cost and/or complexity of relevant animal models for HIV infection currently seem to be slowing the development of these promising systems.

Non-phagocytic 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 (Fig. 2b–e).

Mechanisms of endocytosis

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. For most cell types, CME serves as the main mechanism of internalization for macromolecules and plasma membrane constituents. CME via specific receptor-ligand interaction is the best described mechanism, to the extent that it was previously referred to as "receptor-mediated endocytosis" (RME). However, it is now clear that alternative non-specific endocytosis via clathrin-coated pits also exists (as well as receptor-mediated but clathrin-independent endocytosis).

Notably, the CME, either receptor-dependent or independent, causes the endocytosed material to end up in degradative lysosomes. This has an important impact in the drug delivery field since the drug-loaded nanocarriers may be tailored in order to become metabolized into the lysosomes, thus releasing their drug content intracellularly as a consequence of lysosomal biodegradation.

Receptor-dependent CME 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 [79]. This mode of endocytosis is thus of paramount importance not only for ligands, but also for many viruses (e.g., influenza) [80] and for drug-loaded nanocarriers bearing these targeting ligands on their surface. Numerous ligands have been used for this purpose, including low-density lipoprotein (LDL), transferrin and epidermal growth factor (EGF) [81].

The endocytosis typically occurs in a membrane region enriched in clathrin, a main cytosolic coat protein. Formation of the endocytosis vacuole is driven by assembly of a basket-like structure [82] formed by polymerization of clathrin units (Fig. 4). Clathrin is a three-leg structure called triskelion. These triskelia assemble in polyhedral lattice just on the cytosolic surface of the cell membrane, which helps to deform the membrane into a coated pit of ~150 nm. As the clathrin lattice formation continues, the pit becomes deeply invaginated, until fission of the vesicle occurs, this step requiring the GTPase dynamin, leading to so-called clathrin-coated vesicles. Uncoating of the vesicles later allows recycling of the clathrin units [83]. Some ligands are also recycled, as transferrin and riboflavin [81]. The resulting endocytic vesicle may have an average size of 100 [81] or 120 nm [83]. This vesicle delivers its cargo to "early" (or "sorting") endosomes, which are acidified by ATP-dependent proton pumps (pH ~6) (Fig. 5b). 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 ~5), which, after fusion with prelysosomal vesicles containing acid hydrolases, generate a harsh environment prone to degradation of the internalized cargo [79, 81].

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 [84]. Transcytosis of transferrin is of particular importance

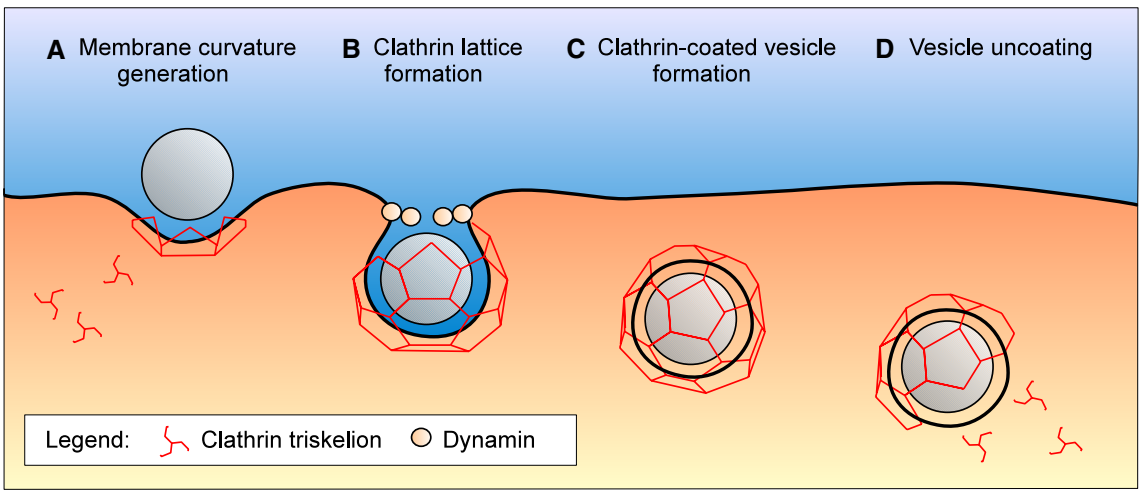

Fig. 4 Vesicle formation during clathrin-mediated endocytosis. **a** The assembly of clathrin triskelions (based on three clathrin heavy chains) into a polygonal lattice helps deform the overlying plasma membrane into a coated pit. **b** After assembly of the basket-like clathrin lattice, dynamin is recruited at the neck of the pit to mediate the membrane fission. **c** This leads to the cytosolic release of the clathrin-coated vesicle. **d** The following uncoating of the vesicle allows the recycling of clathrin triskelia

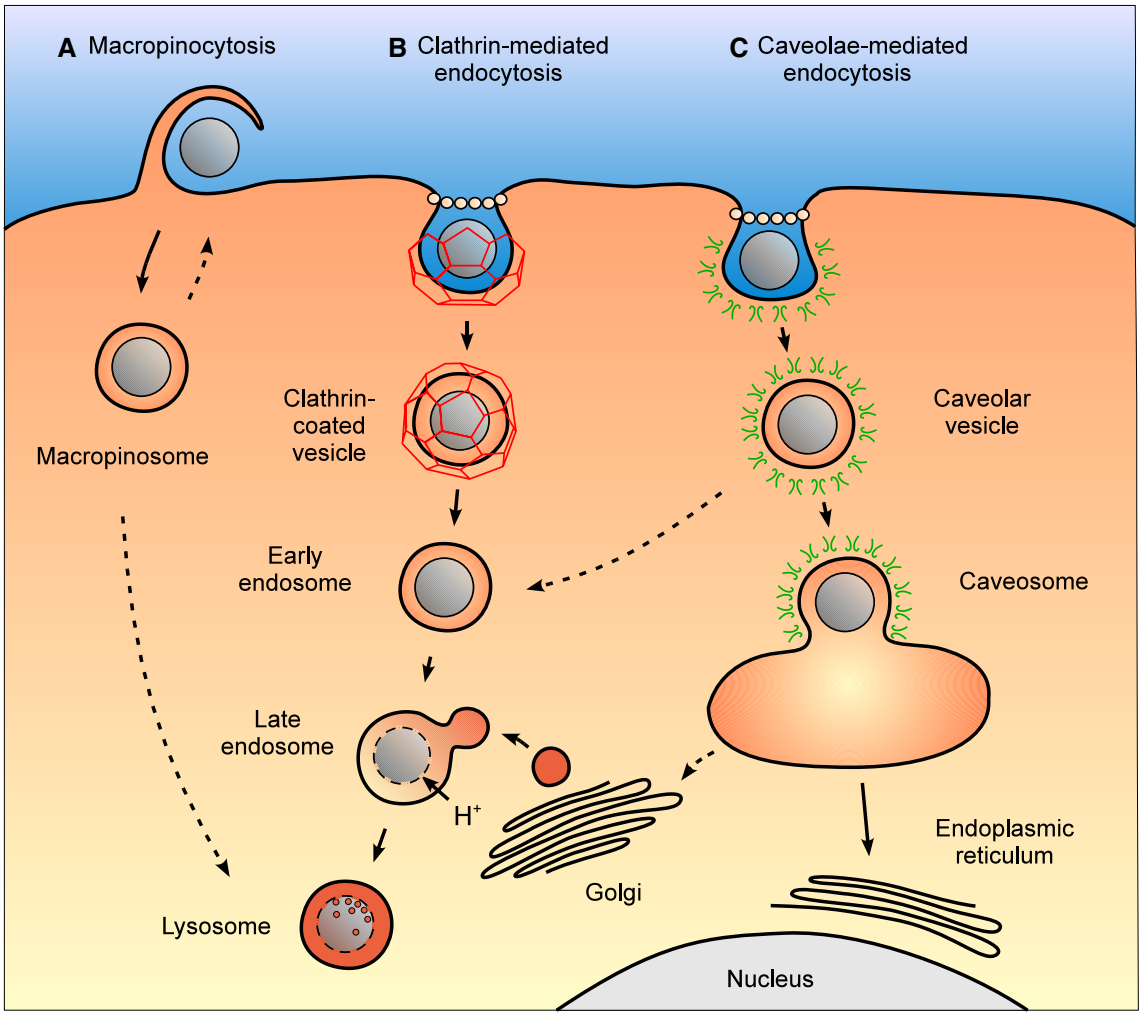

Fig. 5 Intracellular nanocarrier trafficking following macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis. **a** Macropinocytosis leads to the formation of a macropinosome, which is thought to eventually fuse with lysosomes or recycle its content to the surface. **b** Clathrin-mediated endocytosis of a nanocarrier leads to the formation of an early endosome, which is acidified and fuses with prelysosomal vesicles containing enzymes (in red) to give rise to a late endosome and finally a lysosome, an acidic and enzyme-rich environment prone to nanocarrier and drug degradation. Unless a lysosomal delivery is desired, strategies for a cytosolic drug delivery by this route will focus on the drug escape from the endosome as early as possible. **c** Caveolae-mediated endocytosis of a nanocarrier gives rise to a caveolar vesicle that can be delivered to caveosome, avoiding a degradative acidic and enzyme-rich environment

in the case if endothelial cells forming the blood brain barrier (BBB) [85].

Receptor-independent CME Another CME mechanism, involving non-specific adsorptive pinocytosis, has been simply referred to as fluid-phase endocytosis by some authors [81]. Compounds absorbed by this pathway avoid direct binding with membrane constituents, but often display non-specific 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 [81]. Apart from the different mode of interaction with the membrane, the major specificity of this pathway is a slower internalization rate compared to the receptor-dependent CME [86].

Caveolae-mediated endocytosis

Although CME is the predominant endocytosis mechanism in most cells, alternative pathways have been more recently identified, caveolae-mediated endocytosis (CvME) being the major one. Caveolae are characteristic flask-shaped membrane invaginations, having a size generally reported in the lower end of the 50–100 nm range [79, 81, 83, 87], typically 50–80 nm. They are lined by caveolin, a dimeric protein, and enriched with cholesterol and sphingolipids (Fig. 5c). Caveolae are particularly abundant in endothelial

cells, where they can constitute 10–20% of the cell surface [83], but also smooth muscle cells and fibroblasts. CvMEs are involved in endocytosis and trancytosis of various proteins; they also constitute a port of entry for viruses (typically the SV40 virus) [80] 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 [81, 83]. After binding to the cell surface, particles move along the plasma membrane to caveolae invaginations, where they may be maintained through receptor-ligand interactions [81]. Fission of the caveolae from the membrane, mediated by the GTPase dynamin, then generates the cytosolic caveolar vesicle, which does not contain any enzymatic cocktail. Even this pathway is employed by many pathogens to escape degradation by lysosomal enzymes. 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. Ligands known to be internalized by CvME include folic acid, albumin and cholesterol [81] (see below).

Macropinocytosis

Macropinocytosis is another type of clathrin-independent endocytosis pathway [88], occurring in many cells, including macrophages [79]. It occurs via formation of actin-driven membrane protusions, similarly to phagocytosis. However, in this case, the protusions do not zipper up along the ligand-coated particle; instead, they collapse onto and fuse with the plasma membrane [83] (Fig. 2d). This generates large endocytic vesicles, called macropinosomes, which sample the extracellular milieu and have a size generally bigger than 1 μm [83] (and sometimes as large as 5 μm [79]). The intracellular fate of macropinosomes vary 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 [79] (Fig. 5a). Macropinosomes have not been reported to contain any specific coating, nor do they concentrate receptors [89]. This endocytic pathway does not seem to display any selectivity, but is involved, among others, in the uptake of drug nanocarriers.

Other endocytosis pathways

Various clathrin- and caveolae- independent endocytosis pathways 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 [83]. A classification for the clathrin- and caveolae- independent pathways has been proposed only recently [87]. However, the understanding of their implications in the interactions with drug delivery nanosytems is still in a nascent stage.

Nanocarrier characteristics influencing non-phagocytic endocytosis

Contrary to phagocytosis, it is difficult to describe a thorough and consistent profile of nanoparticles 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 will also occur between the apical and basolateral membranes of a polarized cell. 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 PSt nanoparticles (varying from around 20–1,000 nm) were not preferably endocytosed according to their size by the HUVEC endothelial, the ECV 304 bladder carcinoma and the HNX 14C squamous carcinoma 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 [90]. Some trends can however be noted.

Cells from the gastrointestinal epithelium (Caco-2 cell line [91] as well as rat gastrointestinal tissue [92]) display a greater uptake for 100-nm PLGA particles compared to 500 nm–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 [93], as well as for poly(ε-caprolactone) (PCL) particles [94].

The size may also directly affect the mode of endocytosis. It should be pointed out that the typical endosome sizes reported (i.e., 100 nm for CME, 50–80 nm for CvME) [81, 83, 87] do not perfectly match with the sizes of the drug delivery nanoparticles that are most of the time bigger than 100 nm [91, 92, 94]. One possible explanation of this discrepancy is that many investigations on endocytosis mechanisms focused primarily on fundamental biological processes, involving (literally) pinocytosis, i.e., fluid and receptor/ligand pair internalization, rather than solid particles, thus imposing less mechanical constrains on the vesicle formation. Whatever it is, a nice study has exemplified interesting size-dependent endocytosis

pathways in non-phagocytic murine melanoma B16 cells, using 50–1000 nm PSt beads devoid of ligands [95]. 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 of—surprisingly—500 nm in size. Thus, CME was shown to apply to nanoparticles with a size limit of around 200 nm, and kinetic parameters seemed to determine internalization of these particles along CME rather than CvME [95]. More studies are, however, needed 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 PSt nanoparticle internalization by HeLa cells showed that, while beads of 40 nm in diameter entered cells 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 [96], 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 to CME or CvME [97].

In some cases, size may however have little influence on uptake as compared with surface properties (e.g., charge and presence of ligands).

Surface charge

Interaction with the cell membrane Due to the negatively charged character of the cell plasma membrane, drug nanocarriers possessing a positively charged surface 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 [98]) through adsorptive endocytosis, and possibly involving CME. Similar patterns were observed on other A-549 epithelial cells [99]. Other cationic nanocarriers can similarly impact endocytosis. For example, nanoparticles based on PLA-PEG and coated with the cationic lipid stearylamine ($\zeta \approx +35$ mV) showed increased and faster uptake by HeLa cells as compared with the negatively charged parent PLA-PEG nanoparticles ($\zeta \approx -35$ mV), the former using the CME pathway, contrarily to the latter [100].

Interaction with endo/lysosomes The particle electric charge also plays a crucial role in the interaction with the endocytic vesicles, specifically 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 micelle structures at low pH, allowing the fusion between the liposomal and the endosomal membranes, and the destabilization of the endosomes [101]. 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 [102]. Typically, DOPE:OA liposomes become leaky at pH 6.5 and DOPE:CHEMS at pH 5.5 [103]. The transfer of the DOPE molecules to the endosomal membrane is thought to promote endosome leakage [101], although the precise mechanism remains to be elucidated [104]. Such pH-sensitive liposomes have shown efficient in vitro cytosolic delivery of model fluorescent probes [105] and oligonucleotides (ODN) [106]. However, the in vivo efficacy is more questionable, mainly due to stability concerns in the presence of serum [107].

Unlike these pH-sensitive anionic liposomes, lipoplexes, resulting from the complexation of nucleic acids with cationic lipids, exhibit a total net positive charge [104]. They are often designed using the cationic *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [108] and 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP) [109]. 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.

On the contrary, if cationic polymers do not possess fusogenic activity per se, some of them, like the popular poly(ethyleneimine) (PEI), have however the ability to disrupt the endosomal membrane, as a consequence of their important buffering capacity [110]. Indeed, polyplexes resulting from the complexation of DNA plasmids with PEI shown remarkable transfection efficiency on various cell lines, which lead the authors to propose the so-called 'proton-sponge' effect [111]. 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 [111]. Other mechanisms have also been suggested, such as a possible swelling of the polymer network resulting from the

increasing repulsion of the protonated groups [112]. If the precise mechanism of the endo/lysosomal escape as well as the transport to the nucleus is not yet clearly understood, 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 [113].

Interaction with the mucus layer Studying the interaction of nanocarriers with the cell plasma membrane is not sufficient to describe the uptake phenomena by the cellular epithelium. For example, in the gastrointestinal tract, a mucus composed of high molecular weight glycoproteins covers the epithelial cells, which significantly affects nanoparticle uptake by the underlying cells. Thus, the importance of the particle charge has also been also investigated through parallel endocytosis studies by enterocyte-like Caco-2 cells and mucus-secreting MTX-E12 cells [114]. In contrast to Caco-2 cells, the presence of mucus in MTX-E12 cells constituted a major barrier for the uptake of hydrophobic PSt nanoparticles (otherwise efficiently internalized) and also showed a favorable effect upon the uptake of positively charged chitosan nanoparticles [114]. As for the endocytosis mechanisms in the presence of mucus, stearylamine-coated PLA-PEG nanoparticles ($\zeta \approx +30$ mV) were recently shown to enter epithelial kidney cell MDCK (harboring a thin mucus layer) through CME, the positive charge stimulating the movement of nanoparticles towards clathrin-coated pits; a fraction was internalized through a macropinocytotic-like pathway, too [97]. Nanoparticles coated with either chitosan or hyaluroic acid have led to many examples of increased in vivo association to mucosae in the gastrointestinal tract [115], in the nasal mucosa [116] and in the cornea [117].

These trends should not lead to believing that a positively charged surface is a prerequisite for efficient endocytosis. Negatively charged nanoparticles can show efficient uptake, especially when conjugated with targeting ligands, as discussed below.

Non-ionic surface coating

Interaction with the cell membrane Coating nanoparticles with non-ionic polymers like PEG can also influence endocytosis, as suggested by several studies focusing on the interaction between various PEGylated nanoparticles and brain endothelial cells. It was shown that nanospheres prepared from a PEG-poly(hexadecylcyanoacrylate) (PEG-PHDCA) copolymer were able to accumulate in both healthy rat brain and brain glioma 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) [118]. Using an original in vitro model of rat BBB [119], the authors showed that PEG-PHDCAs were internalized through the CME pathway after specific recognition by LDL receptors and accumulated in endosomal/lysosomal compartments [120]. While the total amount of adsorbed proteins was lower on 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 [121]. Similar conclusions were drawn from parallel studies performed on PACA nanoparticles PEGylated by the single adsorption of polysorbate 80 [122]. Although some controversy arose about possible interactions between the desorbed surfactant molecules and the cell tight junctions [123, 124], this was not the case with nanoparticles prepared from the PEG-PHDCA copolymer where the PEG chains are chemically linked, and thus are firmly bound at the surface of the particles.

Interaction with endo/lysosomes The recent development of nanocarriers based on modified PEG has paved the way for new pH-responsive systems, the key feature being the incorporation of acid-labile groups. For instance, polymeric 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 [125]. 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 [126]. 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 [127].

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 of ‘molecular addressing’ 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.

The vitamin folic acid (FA) has been widely studied as a targeting ligand for nanocarriers, especially for anticancer strategies [128]. 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 [129]. In particular, FR is often overexpressed on the surface of cancer cells, but highly restricted in normal tissues [130]. Moreover, FR has the ability to transport both FA and the FA-linked cargo by RME with subsequent endosomal escape into the cytosol [129], thus avoiding lysosomal degradation. Although CvME appears to be involved in the uptake of FA in some cases [131], the complete mechanism is complex and remains debated [129, 132]. FA has been successfully coated onto PEGylated polymeric nanoparticles by conjugation of the activated N-hydroxysuccinimide FA with the aminated PEG-PHDCA copolymer. Plasmon surface resonance revealed that FA bound to these particles had a ten-fold higher apparent affinity for FR compared to free FA [133]. Liposomes were also decorated with FA by incorporating a phospholipid-anchored FA [134] or a FA-PEG-phospholipid conjugate [135] 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 [136], with albumin nanoparticles coated with activated FA [128] and with polymeric micelles prepared from a mixture of poly(L-histidine)-PEG-FA and PLA-PEG-FA [137]. This demonstrates the versatility of FA as a targeting agent applicable to various nanocarriers.

Transferin (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 [138] (typically two- to ten-fold more). PLGA nanoparticles were thereby conjugated with Tf and exhibited a two-fold 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 [139]. 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 [140]. TfRs are also known to be highly expressed in some healthy tissues like brain capillaries where they are known to mediate transcytosis [85]. 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 [85].

However, the use of ligands like Tf for nanoparticle functionalization may be hindered by a competition with the corresponding endogenous pool of ligands [85]. 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 distinct from the Tf binding site, thus preventing competition with endogenous Tf. In this context, OX26 has been conjugated to PEGylated liposomes (so-called immunoliposomes) to increase the brain delivery of the encapsulated drug daunomycin to rats [141]. The transcytosis mechanism of OX26 immunoliposomes was demonstrated using an in vitro model of BBB consisting in a monolayer of rat brain endothelial cells RBE4 [142]. Similar studies were carried out on mice, but using another MAb, the rat 8D3 MAb to the mouse TfR [143]. 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 whose Fc fragment may be recognized by macrophages [144]. Using the avidin (SA)-biotin (BIO) technology, chitosan nanospheres were also conjugated with PEG bearing the OX26 MAb. These functionalized CS-PEG-BIO-SA/OX26 nanoparticles were able to translocate into the brain tissue after intravenous administration [145]. A high density of antibodies at the nanocarrier's surface may, however, increase hydrophobicity and restrict its ability to escape the recognition by the RES, thus limiting possibilities for cell targeting.

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. 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 [146]. The so-called intracellular CAM-1 (ICAM-1) is another particularly interesting target for perturbed endothelial cells. PSt nanoparticles bearing MAb to human and mouse ICAM-1 were developed for this purpose [147]. 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 [148]. The nanoparticles finally trafficked to lysosomes [149]. In vivo studies showed that nanoparticles conjugated with ICAM-1 MAb enabled vascular delivery to pulmonary and vascular endothelium [150].

Cell-penetrating peptides (CPPs), also known as protein transduction domains, have also raised increasing attention due to their ability to translocate across membranes [151]. 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 [152]. An increasing number of examples of conjugation of TAT to liposomes [153], polymeric micelles [154] and polyplexes [155] have been described. However, the internalization mechanism of CPPs remains to be fully elucidated: it may involve macropinocytosis, but also CME and CvME [151], as well as direct penetration [156].

Other ligands have been recently investigated to address nanocarriers to intracellular organelles, like mitochondria and nucleus. While access to the mitochondrial intermembrane is highly restricted by the small size of the voltage-dependent anion channels (3–6 nm) [157], it has been suggested to take advantage of the mitochondrial fusion events [158, 159] to deliver nanoparticles to these organelles. Nucleus targeting of nanoparticles is actively investigated for gene delivery. Promising experiments have been carried out using nuclear localization signal (NLS) peptides [160] or TAT peptides [161] coated onto gold nanoparticles. However, application of this approach to biodegradable nanocarriers is still in a nascent stage.

The decoration of nanocarriers by targeting ligands thus offers a great versatility for the targeting of specific cell populations. However, the understanding of the intracellular fate often remains a challenge, as well as the transposition of in vitro studies to in vivo situations.

Surface multi-functionalization

A more general but challenging approach consists in the design of multifunctional nanosystems. The ultimate goal is to confer simultaneously several features to the nanocarriers to ensure not only cell selectivity, but also efficient internalization, often endosomal escape and even organelle targeting. A recent study describes a promising multifunctional nanosystem. The so-called ‘super pH-sensitive multifunctional polymeric micelles’ were prepared from two copolymers: one being the PEGylated polyhistidine and the other PEGylated polyhistidine-co-PLA functionalized with biotin (Fig. 6a). At pH values of 7.4 (corresponding to normal tissues), the micelles exhibit the PEG chains; at pH between 6.5 and 7.0 (corresponding to the extracellular milieu of most tumors), biotin is exposed on the micelle surface thanks to a pH-sensitive actuator (polyhistidine) that can trigger efficient biotin-mediated endocytosis; at pH below 6.5 (endosomes), the micelle destabilizes, resulting in drug release and disruption of endosomal membrane [162]. A similar strategy has been applied to liposomes coated with PEG using an acid-labile group and also exhibiting the cell-internalization peptide TAT to promote endocytosis (Fig. 6b). At the acidic pH of the tumor tissue, the PEG chains are released allowing the TAT peptides to take off and do the job of entering the targeted cancer cells [163].

Shape

The influence of particle shape on endocytosis has been only recently 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 [164] as well as ICAM-1- and TAT-coated nanoparticles [165]. On the contrary, other studies suggested preferential uptake of rod-shaped [166] or cylindrical [167] particles. Thus, no general tendency can be determined yet. This can be explained by the predominance of other factors like the nature, the size and the surface charge of the nanodevices; multiple endocytic pathways can also be involved simultaneously (CME, CvME, RME, macropinocytosis). The influence of particle shape on the intracellular trafficking also deserves more insight. A recent interesting study has compared layered double hydroxides (LDHs) nanoparticles made of Mg and Al oxides, having hexagonal or rod shapes [168]. 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 [168]. This opens exciting perspectives, especially for the control of the intracellular gene delivery.

Drug delivery applications

Delivery of cytotoxic agents

One of the main successes in the use of nanocarriers for the delivery of a cytotoxic drug to tumors located outside of the RES is the formulation of doxorubicin using PEGylated liposomes, approved as Doxil® for the treatment of some ovarian cancers [169]. This breakthrough originates in the ability of these liposomes to escape phagocytosis and extravasate selectively through the fenestrated and leaky vasculate that generally characterize tumor vessels (known as enhanced permeability and retention effect) [170].

However, the development of nanomedicines based on the control of nanocarriers endocytosis is currently still in an earlier stage, although promising in vivo studies have already been reported. Progress in the understanding of

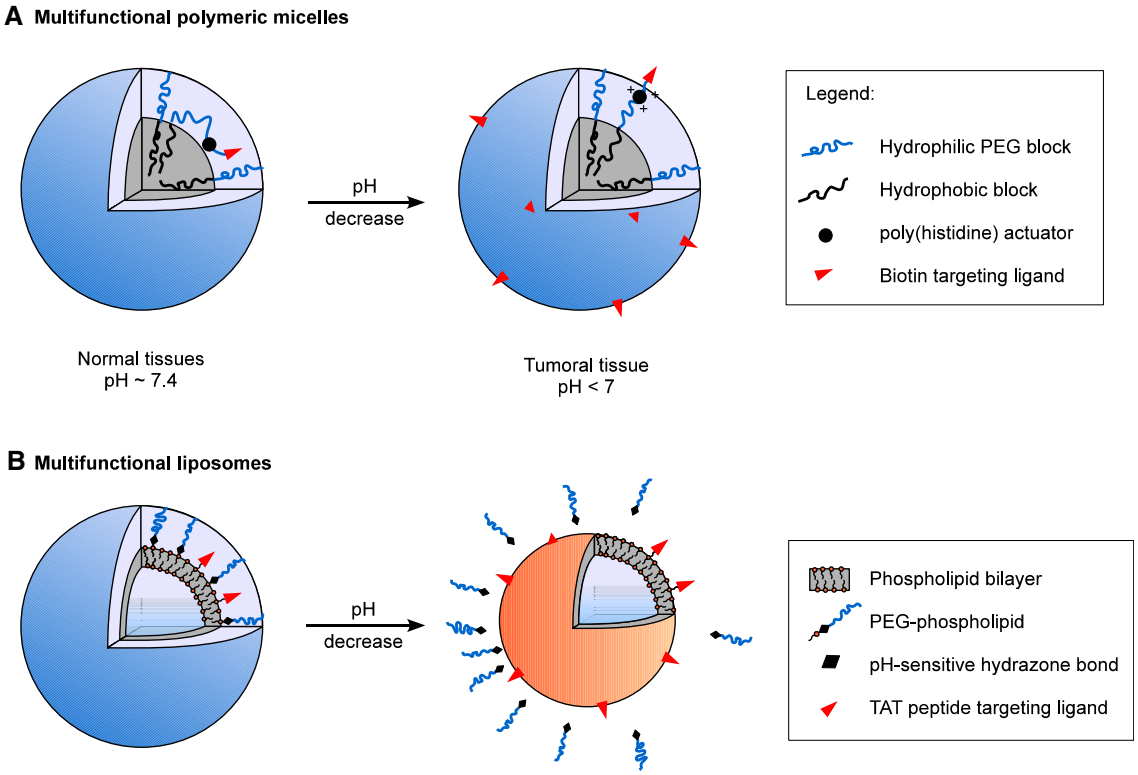

Fig. 6 Examples of multifunctional nanocarriers. Such systems, also called "double-targeted," can exhibit different surface properties depending on the external stimuli. In particular, their hydrophilic PEG coating allows a prolonged circulation time in normal tissues (where pH ~7.4) and a specific extravasation in tumoral tissue through the EPR effect; but once in the tumoral tissue (where pH decreases to around 6.5–7), the targeting ligands get the upper hand and promote nanocarrier internalization in the target cells. **a** Multifunctional polymeric micelles can be formulated using a pH-sensitive poly(histidine) actuator. Unionized, this actuator maintains the biotin ligand close to the hydrophobic core, hidden within the PEG chains. Once ionized, the actuator allows the exposition of biotin out of the PEG chain [162]. **b** Multifunctional liposomes can be PEGylated using an acid-labile hydrazone bond, which releases the PEG chains in an acidic medium, thus exposing the TAT targeting ligands coated directly on the surface of the liposomes [163]

PEGylated nanoparticle interaction with BBB endothelial cells has led to the development of PBCA nanoparticles coated with polysorbate 80 and loaded with doxorubicin for the targeting of glioblastoma. Significant rat survival rates have been obtained with this system [171].

The use of targeting ligands adds a level of complexity, but offers a great potential of versatility to treat various cancers. Along this line, FA-conjugated liposomes efficiently delivered doxorubicin to cancer cells in a murine xenograft model [172]. Tf-conjugated liposomes also proved their capacity to increase the targeting of doxorubicin to implanted colon tumor cells in mice through specific RME [173]. Targeting peptides like RGD were also coupled to liposomes and showed superior anticancer efficacy on a colon cancer xenograft model [146]. Longer peptide sequences, coupled to a N-(2-hydroxypropyl)methacrylamide (HPMA)—doxorubicin conjugate were also successfully used to target the Hsp47/CBP2 chaperone protein, a surface-specific receptor overexpressed in human squamous cell carcinoma of the head and the neck [174]. The use of antibodies as targeting agents has also shown a promising efficacy in the treatment of other experimental cancers. For example, anti-CD19 immunoliposomes were successfully addressed to circulating B-lymphoma cells through specific recognition of their CD19 receptors [175]; anti-HER2 immunoliposomes were able to deliver doxorubicin to breast cancer xenografts better than PEGylated liposomes [176]. The Fab' fragment of anti-HER2 has also been recently coupled to PLGA nanoparticles, which enhanced the anticancer activity and decreased the toxicity of a model protein toxin in a tumor xenograft murine model [177].

Delivery of nucleic acids

Due to their poor intracellular penetration, the therapeutic use of nucleic acids heavily relies on appropriate drug delivery systems, either in case of gene therapy using DNA (needed to be delivered into the cell nucleus) or in the case of an antisense strategy using oligonucleotides (ODN) or short-interfering RNA (siRNA) (needed to be delivered into the cell cytoplasm). The nanocarriers that have been

developed for nucleic acid delivery often rely on cationic lipids or polymers that form lipoplexes or polyplexes by engaging electrostatic interactions with DNA, ODN or siRNA (Table 1).

Cationic lipids, also called cytofectins or lipofection reagents, have been widely used to formulate liposomes for gene delivery. Since the first use of DOTMA for in vitro DNA transfection of various cell lines [178], numerous liposomes have been synthesized and used for the delivery of nucleic acids in animals as well as in patients enrolled in phase I and II clinical trials [179], mainly for the treatment of cystic fibrosis (CFTR gene transfer) [180] and cancer (e.g., HLA-B7 gene transfer) [181]. However, a relatively low transfection efficiency as well as toxicity appeared to be important limitations of these formulations.

Alternative polymer-based cationic nanocarriers have received increasing attention [182]. Polyplexes formed by complexation of PEI and nucleic acids have shown efficient in vitro delivery of DNA [111], ODN [183] and siRNA [184] in various cell lines. In vivo, these polyplexes also displayed antitumoral activity after subcutaneous or intraperitoneal administration, as shown with a siRNA targeted against the growth factor pleiotrophin [185] and the HER-2 receptor [186] in murine subcutaneous tumor models. In a recent phase I/II clinical trial on patients with superficial bladder cancer, intravesicular administration of the BC-819 plasmid DNA complexed with PEI demonstrated evidence of tumor destruction by complete ablation of marker tumors [187]. PEGylated polyplexes have been used to target various organs using the intravenous route of administration. Unfortunately, the presence of the PEG chains was found to shield the surface charge of the polyplexes, thus decreasing their cellular uptake; however, conjugation of an RGD peptide at the extremity of the PEG chains was found to restore the uptake level of the original polyplexes, but this time through specific RME [188]. These RGD-PEG polyplexes allowed efficient in vitro luciferase gene inhibition by siRNA, as well as in vivo accumulation in cancer cells, after intravenous administration to mice. Tumor growth inhibition was also observed when using a siRNA targeted against the vascular endothelial growth factor receptor-2 [188]. Despite these promising results, the usefulness of PEI is often limited by its toxicity and non-biodegradability.

The loading of ODN onto the biodegradable PACA nanoparticles was found possible by using cationic surfactants like cetyltrimethylammonium bromide (CTAB), which, when pre-adsorbed onto the surface of these nanospheres, permitted the formation of ion pairs with the ODNs molecules [189]. These nanoparticles were found to increase dramatically the cellular uptake of the ODN; they also inhibited mutated Ha-*ras*-mediated cell proliferation and tumorigenicity after subcutaneous administration to mice [190]. A recent development of this technology consisted in the preparation of nanoparticles composed of a PIBCA core and a chitosan brush-like shell, able to bind siRNA on their surface (chitosan is considered less toxic than CTAB). After subcutaneous administration, this nanosystem was well tolerated and significantly reduced the tumor size in an experimental model of papillary carcinoma of the thyroid through specific inhibition of the ret/PTC1 junction oncogene target [191]. The fine tuning of the surface properties allowed by the original synthesis of these nanoparticles holds some promise for the control of their interactions with target cells.

The use of cationic systems remains, however, problematic in vivo. Their interaction with negatively charged serum protein and red blood cells may, indeed, form large clusters [192], whereas inside the cell, cationic polyplexes may interfere with normal metabolic processes [193]. Therefore, attention should be paid to the possibility of shifting towards non-cationic biodegradable polymeric nanocarriers for the delivery of nucleic acids. An original approach consisted of the synthesis of PIBCA nanocapsules containing an aqueous core, able to efficiently entrap ODN [194] and siRNA [195]. These nanocapsules dramatically increased the cell uptake of the nucleic acids, allowing their delivery to the cell cytoplasm and nucleus [196]. A significant tumor growth inhibition was observed on a murine model of Ewing sarcoma after intratumoral administration of these nanocapsules loaded with an ODN [197] or a siRNA [195] targeted against the EWS Fli-1 fusion oncogene, whereas the naked ODN or siRNA had no effect.

Delivery of peptides and proteins

Nanotechnology has a great potential to improve the delivery of peptides and proteins, especially for the treatment of major metabolic diseases. Indeed, current treatments of type 1 diabetes and osteoporosis rely on the parenteral administration of insulin and calcitonin, respectively. Alternative non-invasive routes of administration, such as the oral, nasal and pulmonary ones, offer the promise of improved compliance and safety of treatments, but are hampered by the inability of these macromolecules to efficiently cross mucosal barriers.

Nanocarriers have thus been extensively investigated for the oral delivery of insulin. Unlike liposomes, whose stability was generally insufficient to withstand the harsh conditions of the gastrointestinal tract [198], polymeric nanoparticles offered a good protection of the encapsulated drugs and were efficiently absorbed across the intestinal epithelium [199]. Remarkably, PIBCA nanocapsules loaded with insulin induced a reduction of glycemia in diabetic rats for up to 20 days [200]. They were shown to

Table 1 Internalization pathways of major nanocarriers for drug delivery

Nanocarrier	Drug	Main internalization pathways	In vitro models	In vivo models	Remarks	References
PSt-based nanoparticles						
	–	Phagocytosis	Rat/mouse Mac, *Acanthamoeba*	–	Nanoparticle size and shape influence phagocytosis.	[19, 56]
	–	CME, CvME	Mouse melanoma B16 cells	–	CME predominant for nanoparticles below 200 nm, CvME involved above	[95]
PACA-based nanoparticles						
Conventional	Doxorubicin	Phagocytosis (Mac) or non-endocytotic (cancer cells)	MDR cancer cells	Metastasis-bearing mice	Liver Kupffer cells act as a drug reservoir. Transdrug® in phase II/III clinical trials for hepatocarcinoma	[57–63]
Conventional	Azidothymidine	Phagocytosis	Human Mo/Mac	Rats	Targeting of Mac of the RES. Uptake is increased by cell infection by HIV	[20, 75–77]
PEGylated	Doxorubicin	RME *via* LDL receptors	Rat brain endothelial cells	Glioblastoma-bearing rats	Preferential accumulation in brain after BBB crossing	[118, 120–124, 171]
Polyester-based nanoparticles						
PLA, PLGA	Plasmid DNA	Various, including CME	Vascular smooth muscle cells, MCF-7 and PC-3 cancer cells	–	Uptake and endosomal escape are influenced by surface-associated surfactants	[104]
PLA-PEG	–	CME, macropinocytosis	MDCK epithelial cells	–	Cationic surface avoid lysosomal degradation	[97, 100]
PLGA-PEG-Tf	Paclitaxel	TfR-mediated endocytosis	MCF-7 cancer cells	Solid tumor-bearing mice	Greater uptake and reduced exocytosis result in paclitaxel increased activity	[139, 140]
Chitosan-based/coated nanoparticles						
	Proteins	CME, adsorptive endocytosis	Caco-2, mucus-secreting MTX-E12, A-549 epithelial cells	Rats	Chitosan confers mucoadhesive properties to nanoparticles	[98, 99, 114, 207]
Liposomes						
Conventional	–	Phagocytosis	Mouse Mac	Rats	Uptake influenced by liposome size, composition, and rigidity	[23, 24, 29]
SUV	Amphotericin B	Phagocytosis	Mac, Langerhans cells, fungi	Mice and rabbits infected by fungi and leshmania	AmBisome® is marketed for the treatment of leshmaniasis and various intracellular fungal infections	[41, 66, 67]
pH-Sensitive	ODN	Endocytosis followed by endosomal escape	CV-1, psi2*neo*, 3T3 cells	Mice	Cytosolic delivery	[101–107]
Polyplexes						
PEI-based	DNA, ODN, siRNA	Endocytosis followed by endosomal escape	3T3, HepG2, COS-7, HeLa, neurons	Mice	Polyplexes escape endosomes through "proton sponge" effect. Phase I/II clinical trials	[111-113, 186, 188]

BBB blood-brain barrier, *CME* clathrin-mediated endocytosis, *CvME* caveolae-mediated endocytosis, *Mac* macrophage, *MDR* multi-drug resistant, *Mo* monocyte, *ODN* oligonucleotide, *PACA* poly(alkylcyanoacrylate), *PEG* poly(ethyleneglycol), *PEI* poly(ethyleneimine), *PLA* poly(lactic acid), *PLGA* poly(lactic-*co*-glycolic acid), *PSt* polystyrene, *SUV* small unilamellar vesicle, *Tf* transferrin, *TfR* transferrin receptor

be absorbed through paracellular or transcellular pathways by enterocytes (and Goblet cells), but were degraded in M-cell-rich regions of the epithelium [201]. This system, however, induced a variable response when tested in dogs [202], which has limited its development. The coating of nanoparticles with hydrophilic polymers, such as PEG [203], chitosan [116] and cationic polyacrylic polymers such as Eudragit® RS [204], was also found to provide efficient in vivo transport of proteins through preferential interaction with the mucus (mucoadhesion), and, in some cases, through the opening of tight junctions and paracellular transport. To date, however, many questions are still unanswered regarding interactions of nanoparticles with the various cell populations of the intestinal mucosa, and the delivery of a reproducible insulin dose by the oral route remains a challenge.

Nanoparticles were also designed for the delivery of peptides through the nasal mucosa. For example, chitosan nanoparticles loaded with insulin were able to significantly and relatively rapidly reduce glycemia after nasal administration to rabbits compared to insulin mixed with soluble chitosan [205]. Here also, the coating of nanoparticles with PEG and chitosan was found to increase the transport across the nasal mucosa, as demonstrated for PEGylated PLA nanoparticles [206] and chitosan-coated nanocapsules [207]. Although the mucoadhesive properties of chitosan seem to be determinant for the in vivo efficacy of these systems, more investigations are needed to better characterize the nanoparticle uptake through the nasal mucosa, as well as its implications in terms of toxicity.

The pulmonary route has also received much attention for the delivery of peptides [208]. Administered by this route, PLGA nanoparticles loaded with insulin have shown interesting hypoglycemic effects comparatived to free insulin in a guinea pig model [209], as PBCA nanoparticles did in rats [210]. Chitosan-coated PLGA nanoparticles enhanced the absorption of the calcitonin peptide when delivered to the lungs of guinea pigs, which was attributed to a mucoadhesive effect and a possible opening of tight junctions [211]. Unlike these formulations, delivered by nebulization of a colloidal suspension, the introduction of "porous nanoparticle-aggregate particles" (PNAPs) has allowed the delivery of nanoparticles as a dry powder. These structures are formed by the assembly of nanoparticles into hollow or porous micron-scale particles, thus combining the stability and inhalation efficiency of dry microparticles, and the drug delivery potential of nanoparticles that are released upon exposition of the PNAPs to the lung lining fluid [212]. Chitosan nanoparticles loaded with insulin were formulated as PNAPs, suited for pulmonary administration [213]. Mucoadhesion of these nanoparticles was demonstrated on the Calu-3 and A549 respiratory endothelial cells, but not internalization [214]. Thus to date, more investigations are needed to understand the interactions between PNAPs and lung epithelial cells.

Conclusion

Progress in understanding the nanoparticle internalization by a variety of mammalian cells has already allowed the design of effective nanomedicines, especially for the treatment of infectious diseases and some cancers. However, the most advanced applications often rely on phagocytosis, while the complexity of targeting the other endocytic pathways is highlighted by the difficulties of tailoring nanocarriers able to reproducibly cross various mucosa. 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 expanding knowledge about biological markers offers increasing possibilities to target nanocarriers to the desired cell populations. New multifunctional nanocarriers are also emerging that are able to target cells in vivo, and also to optimize the cellular uptake and to control the intracellular fate. However, the major challenge remains of combining the increasing complexity of always more efficient drug nanocarriers with the need to minimize their potential toxicity and maybe also to rationalize their conception with a view to providing widely shared delivery platforms.

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