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EXPERT REVIEW

Barriers to Non-Viral Vector-Mediated Gene Delivery in the Nervous System

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ABSTRACT Efficient methods for cell line transfection are well described, but, for primary neurons, a high-yield method different from those relying on viral vectors is lacking. Viral transfection has several drawbacks, such as the complexity of vector preparation, safety concerns, and the generation of immune and inflammatory responses when used *in vivo*. However, one of the main problems for the use of non-viral gene vectors for neuronal transfection is their low efficiency when compared with viral vectors. Transgene expression, or siRNA delivery mediated by non-viral vectors, is the result of multiple processes related to cellular membrane crossing, intracellular traffic, and/or nuclear delivery of the genetic material cargo. This review will deal with the barriers that different nanoparticles (cationic lipids, polyethyleneimine, dendrimers and carbon nanotubes) must overcome to efficiently deliver their cargo to central nervous system cells, including internalization into the neurons, interaction with intracellular organelles such as lysosomes, and transport across the nuclear membrane of the neuron in the case of DNA transfection. Furthermore, when used *in vivo*, the nanoparticles should efficiently cross the blood-brain barrier to reach the target cells in the brain.

KEY WORDS delivery · gene therapy · membrane crossing · nanoparticles · neurons

INTRODUCTION

Gene therapy can be defined, in a broad sense, as an attempt to introduce effective genes into malfunctioning target cells from patients suffering from diseases of genetic origin or to interfere with signaling pathways involved in the genesis of different diseases by selectively knocking down different proteins using RNA interference technology.

This approach has been shown to be especially useful in tumoral cell lines (1). Neurons, however, are difficult to transfect, and initial strategies to deliver genetic material to neurons have relied on the use of viral vectors. This approach has several drawbacks, especially safety concerns and the possibility of an immune response against the viral agents employed.

The use of non-viral vectors has been proposed to overcome the aforementioned drawbacks of their viral

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counterparts. However, one of the main problems of such non-viral gene vectors is their low transfection efficiency compared with viral vectors (2, 3). Transgene expression, or siRNA delivery mediated by non-viral vectors, is the result of multiple processes related to cellular membrane crossing, intracellular traffic and/or nuclear delivery of the genetic material cargo.

This review will deal with the barriers that different nanoparticles (NPs) (cationic lipids, polyethyleneimine, dendrimers and carbon nanotubes) must overcome to efficiently deliver their cargo to central nervous system (CNS) cells, including a) internalization into the neurons, b) interaction with intracellular organelles such as lysosomes, and c) transport across the nuclear membrane of the neuron in the case of DNA transfection. Furthermore, when used *in vivo*, the nanoparticles should efficiently cross the blood-brain barrier (BBB) to reach the target cells in the brain.

BARRIERS TO CELLULAR UPTAKE

Neuronal cells have special properties, such as their polarized nature and the elongated morphology of neuronal projections, which could result in barriers to uptake that are unique to such cells (4). Fortunately, however, the surface of nanomaterials can be designed to obtain specific physical or biological properties, thereby allowing specific interactions with cytoplasmic membranes and improving this uptake. The internalization pathway includes two subtypes, namely phagocytosis and pinocytosis (Fig. 1).

Non-viral vectors can take advantage of multiple uptake pathways simultaneously in order to become internalized in cells, although some of these lead to degradation when uptake is higher than the efficiency of the treatment. However, this diversity of uptake pathways makes the use of NPs to deliver therapeutic molecules into neuronal cells possible (4).

Phagocytosis

Nanoparticle uptake mechanisms are different for neurons and glia. Thus, in macrophage-like cells, such as microglia, the internalization of nanocarriers up to 10 μm in diameter depends heavily on phagocytic activity, a process that does not take place in neuronal cells (5). Phagocytosis plays an important physiological role in the clearance of apoptotic cells by preventing the efflux of potentially harmful components from dying cells, thereby limiting direct tissue injury, and by inhibiting secondary immune responses (6). In addition, microglial phagocytosis represents a first line of defense against pathogens in the CNS.

The composition of NPs plays a critical role in phagocytosis, and functionality at the surface of the nanocarriers seem to be the most important driving force for phagocytosis, although other characteristics of the NPs, such as their rigidity and shape, may also have an effect on phagocytosis (7).

When NPs are used as non-viral delivery systems, the main role of the cells involved in phagocytosis in the CNS is to clear NPs from the brain. This internalization pathway therefore plays a critical physiological role by reducing the toxicity and effectiveness of most NPs used for drug or genetic material delivery. Additionally, degradation by macrophages also results in degradation of these nanocarriers before they reach the CNS (8).

Pinocytosis

Non-phagocytic endocytosis, traditionally referred to as pinocytosis, forms part of the whole cell membrane flux

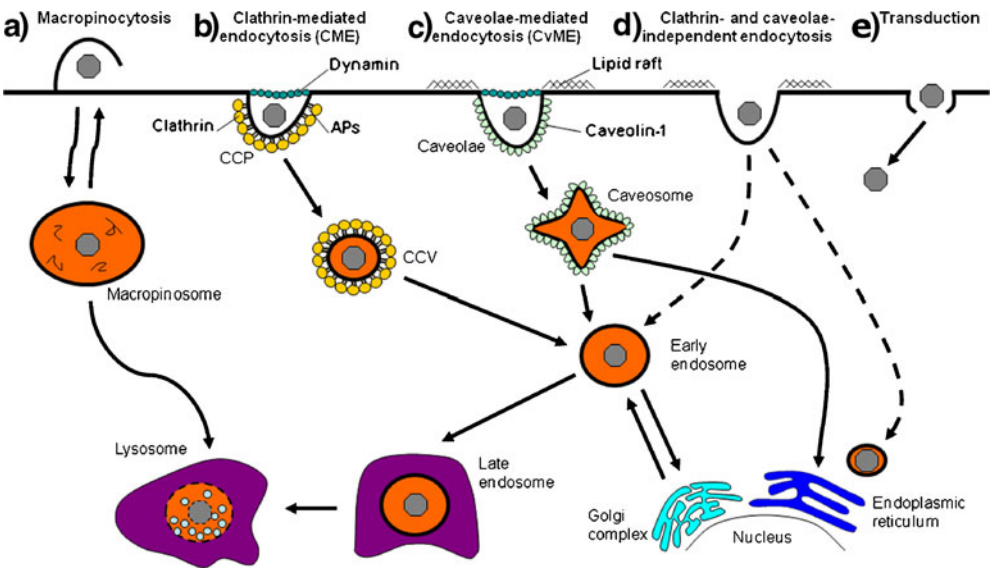

Fig. 1 Scheme representing the different mechanisms of nanoparticle (○) internalization, including (**a**) macropinocytosis, (**b**) clathrin-mediated endocytosis, (**c**) caveolae-mediated endocytosis, (**d**) clathrin and caveolae-independent endocytosis and (**e**) transduction. *Dashed lines* represent alternative pathways.

that facilitates communication between the cell and the extracellular environment. This process allows cells to internalise molecules from the external environment for metabolic purposes, recycling or degradation in lysosomes. Pinocytosis, together with exocytosis, therefore contributes to the cellular membrane balance. Due to their small size, solid NPs can be internalized via these non-phagocytic pathways. Unlike phagocytosis, which is restricted to specialized cells, these endocytic pathways can be conducted by almost every cell type, including neurons.

Types of Pinocytosis

There are two major types of pinocytosis: fluid-phase endocytosis and receptor-mediated endocytosis.

Fluid-phase Endocytosis. Fluid-phase endocytosis (FPE) is a non-specific mechanism used for the uptake of compounds contained in the extracellular fluid. The main characteristic of this process is its lack of selectivity. It results in the uptake of the extracellular fluid surrounding the cell by means of an invagination of the plasma membrane to form an endocytic vesicle. The attachment of molecules to the cell surface takes place by means of non-specific mechanisms such as electrostatic interactions, hydrogen bonding or van der Waals forces, amongst others (9). Cationic molecules interact with the negatively charged plasmatic membrane, thereby facilitating their internalization. This has led to the proposal that cationization might be a strategy to enhance drug delivery to the CNS (10). Indeed, the most successful strategies for delivering genetic material to the CNS have involved the use of positively charged NPs.

Receptor-Mediated Endocytosis. This process is fully specific and takes advantage of the high affinity binding constants between the receptors and their specific ligands. Two different mechanisms have been defined, namely constitutive (class I) receptor-mediated endocytosis (RME), which involves internalization of the ligand bound to its receptor without the generation of any signal, and ligand-stimulated (class II) RME, which involves internalization of the ligand-receptor complex following the generation of signalling molecules. CNS cells are highly differentiated from other body cells. This differentiation also results in the existence of CNS-specific receptors. Neurons express a number of different classes of receptors, including neuropeptide, neurotrophin and neurotoxin receptors (4), thereby leading to strategies that are focused on brain drug-delivery that take advantage of the high specificity of ligand-receptor binding. The transferrin (Tf) or low-density lipoprotein (LDL) receptors are among those defined as constitutive (class I) receptor-mediated endocytosis, whereas others, such as insulin or epidermal growth factor (EGF), are referred to as ligand-stimulated (class II) receptor-mediated endocytosis.

Molecular Mechanisms of Endocytosis

Clathrin-Mediated Endocytosis. Clathrin-mediated endocytosis (CME) via clathrin-coated pits (CCPs) is the best characterized pathway for cellular uptake (Fig. 1). The CME pathway represents a common route of cellular uptake for internalized ligands (11) and viruses (12), as well as NP-based drugs or genetic material delivery systems (13). NPs predominantly use this pathway for the intracellular delivery of active molecules through receptor-mediated endocytosis, such as those produced by Tf, low-density LDL and EGF (14).

During CME, the membrane forms invaginations that are coated by the protein clathrin. The GTPase dynamin, along with other proteins, is mainly responsible for the detachment of CCPs from the cell membrane.

CCPs also contain a high number of receptors that will be involved in different interactions, such as lipid-lipid, lipid-protein or protein-protein (15). These interactions determine the vesicle function and target (16).

Receptor-mediated endocytosis provides the potential for high selectivity in cellular targeting. The cellular membrane expresses a limited number of specific receptors, which can be used to bind NPs whose surface is modified with receptor-specific ligands, thereby resulting in internalization of the ligand and its appended NP via endocytosis.

CME, whether receptor-dependent or -independent, is thus very important for drug-loaded nanocarriers, which have to release their cargo intracellularly. CME is very active in the nervous system, where it is responsible for synaptic vesicle retrieval after nerve stimulation in the retina (17), in either central nerve termini or neuromuscular preparations following large non-physiological stimuli (18, 19) or after trains of action potentials within the physiological range (20).

Caveolae-Mediated Endocytosis. Caveolae-mediated endocytosis (CvME) also plays a role in cellular uptake of different compounds (Fig. 1). Lipid rafts have been defined as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched membrane domains that compartmentalise cellular processes (15, 21). The endocytic process that involves caveolae budding is defined as a clathrin-independent pathway. Ligands known to be internalized by CvME include folic acid, albumin and cholesterol (15).

The CvME pathway has received increasing attention for drug-delivery applications using nanocarriers (12). Folic acid, or vitamin B9, appears to be an attractive target for NP-mediated drug or nucleic acid delivery using this mechanism.

Designed nanocarriers exploiting CvME may be employed to prevent degradation by lysosomal enzymes when the cargo carried (e.g., peptides, proteins, nucleic acids, etc.) is highly sensitive to lysosomal enzymes. In addition, this mechanism might play an active role in transcytosis (transport across a cell via endocytosis at one side and exocytosis at the other) of specific membrane proteins and ligands in endothelial cells (22) and therefore might play a role in NP transcytosis and crossing of the BBB (23).

Lipid rafts play important roles in neural tissue and have been shown to be involved in growth factor signaling by tyrosine kinase receptors (24), axonal guidance (25), ionic channels traffic (26) and recycling of receptors (27).

Non-clathrin, Non-caveolae-Mediated Endocytosis. Various clathrin- and caveolae- independent endocytosis pathways have been described and classified only recently (21) (Fig. 1). However, the understanding of their involvement in NP-mediated drug delivery is still at a nascent stage, although it has been suggested than this mechanism is present in the CNS (28).

Macropinocytosis. Macropinocytosis is another type of non-clathrin, non-caveolae endocytic pathway (29) that can occur in all cell types at different rates (30) (Fig. 1). Macropinocytosis refers to the generation of large endocytic vesicles (up to 5 μm in diameter), which is associated with the formation of actin-dependent membrane ruffles (29). This uptake mechanism shows poor selectivity and is involved in the uptake of NPs (31). In some cases, the size of the NPs has a much lower influence on uptake than their surface properties (e.g., charge and presence of ligands). Macropinocytosis is an attractive pathway to explain the uptake of large NPs in both glial and neural cells.

Non-endocytotic Pathway/Transduction. A non-endocytotic pathway, called transduction, for the taking-up of NPs occurs in many cell types and even in artificial unilamellar vesicles (32) (Fig. 1). However, few efforts have aimed to study this mechanism. There are two models proposed to explain it: the "direct membrane penetration" model and the "inverted micelle" model (32, 33), both of which propose a three-step internalization process involving membrane interaction, membrane permeation and release of NPs into the cytosol.

LYSOSOMES, A CRITICAL BARRIER FOR NANOCARRIERS

Lysosomes are probably the most important barrier to genetic material delivery to different cell types, including neurons. Following endocytosis, the vacuoles become accessible to early endosomes, before fusing with late endosomes and, finally, with lysosomes to form a phagolysosome (34). The lysosomal environment, which is characterized by a low pH and the presence of hydrolytic enzymes, can rapidly degrade a broad range of NPs and their attached cargos, including DNA, RNA, many proteins and therapeutic agents. It is, therefore, not surprising that endosomal escape has been identified as a major limiting step for the effectiveness of NPs in the CNS (35).

Differences in intracellular trafficking can occur mainly based on different uptake mechanisms, so the clathrin-dependent RME pathway is linked to lysosomal degradation, while the clathrin-independent RME internalization favours endosomal accumulation and sorting to a non-degradative pathway (36). NPs internalized by endocytosis are therefore enclosed in vesicles from which they must escape to find the target sites of their cargos before they enter the degradation pathway of the lysosome.

Different endosome escape mechanisms, such as a possible swelling of the NPs resulting from an increased repulsion between the protonated groups, have been suggested (37). Indeed, NPs resulting from the complexation with poly(ethyleneimine) (PEI) showed remarkable transfection efficiency in various cell lines, leading to the proposal of the so-called "proton-sponge" effect (38). According to this hypothesis, the decrease in endosomal pH results in a high protonation of PEI and therefore an osmotic swelling and subsequent vacuole disruption, releasing the NPs and their cargo (38). Although the precise mechanism of the endo/lysosomal escape is not yet clearly understood, PEI-based NPs have been found to perform better than other types of NPs. Indeed, they remain one of the best transfection agents due to the ability to escape from the endosomes. In addition to PEI, other ligands such as DOPE (dioleoylphosphatidylethanolamine) molecules and some thiol groups have been shown to promote leakage from the endosomes (39). Moreover, other strategies to improve lysosomal escape have been used. Thus, NPs linking together two different kinds of RNA, namely pRNA (packaging RNA), which acts as the delivery vehicle, and siRNA, which acts as the therapeutic agent, have been generated (40). Finally, a recent study has described a promising multifunctional nanosystem known as "super pH-sensitive multifunctional polymeric micelles" (41).

Some additives can promote lysosomal escape in neuronal cells. Thus, when SH-SY5Y and N18-RE105 neuronal hybrid cells were transfected in the presence and

absence of chloroquine, transfection efficiencies in the presence of chloroquine were enhanced 600- and 250-fold, respectively (42). Furthermore, some studies have determined that positive charges on the surface of NPs are important to stimulate efficient uptake and endosomal escape, and that other surface modifications such as arginine are also important for an efficient escape (43).

NUCLEAR DELIVERY OF NANOCARRIER CARGOS

The nuclear targeting of NPs is an active area of research in the field of gene delivery. Although it has been reported that some naked DNAs can diffuse from the cytosol to the nucleus across the nuclear membrane, it is hard for large DNA molecules to cross the nuclear membrane while cytoplasmatic nucleases are contributing to DNA degradation, thereby reducing transfection efficiency (44). Moreover, it has been reported that less than 1% of plasmid DNA (pDNA) introduced into the cytoplasm eventually reaches the nucleus, thus resulting in poor gene expression (45), whereas the injection of pDNA directly into the nucleus results in high gene expression.

The nucleus is surrounded by a double membrane which contains highly regulated transport structures called nuclear pores (NPC). There seem to be three possible routes for DNA entry into the nucleus: i) entry during mitosis when the nuclear envelope breaks down, ii) transport through nuclear pores, and iii) active transport across the nuclear membrane by using kariophilic proteins as transfer carriers (Fig. 2).

Nucleocytoplasmic trafficking allows the free diffusion of cargo molecules (plasmid DNA or gene-regulating proteins, oligonucleotides and peptides) up to 9 nm in diameter (about 50 kDa). On the other hand, active transport requires the presence of a nuclear localization signal (NLS) in the cargo that binds to nuclear import machinery and subsequently translocates across the NPC (46).

In constantly dividing cells, the nuclear membrane breaks down at the end of each mitosis, thus allowing the passive inclusion of transfected DNA. However, in non-dividing cells, including post-mitotic neuronal cells, in which mitotic activity is absent, low pDNA nuclear translocation occurs, probably as a result of passive movement through the NPC or by fusion of lipoplexes with the nuclear membrane (47) (this amount being negligible). The primary barrier to DNA transfection in post-mitotic cells is thus assumed to be DNA translocation to the nucleus (48), and several strategies have been described to improve nuclear delivery. Different NPs therefore have different abilities to deliver their cargo to the nucleus.

Cationic Lipids

Cationic lipids, which are commonly comprised of a polar headgroup and non-polar symmetric or dissymmetric carbon-based tail, condense and protect nucleic acids from degradation in the extracellular environment (49) (Fig. 3).

Once lipoplexes escape from the endosome, the pDNA appears to dissociate from the cationic lipid and moves uncoated through the cell into the nucleus. Several studies have shown that high levels of reporter gene expression by lipofection are only obtained in actively dividing cells progressing through the cell-cycle M phase (50).

It has also been postulated that pDNA nuclear entry could also occur by passive diffusion or energy-dependent passage through the NPC (51). Transfection of NIHT3 with

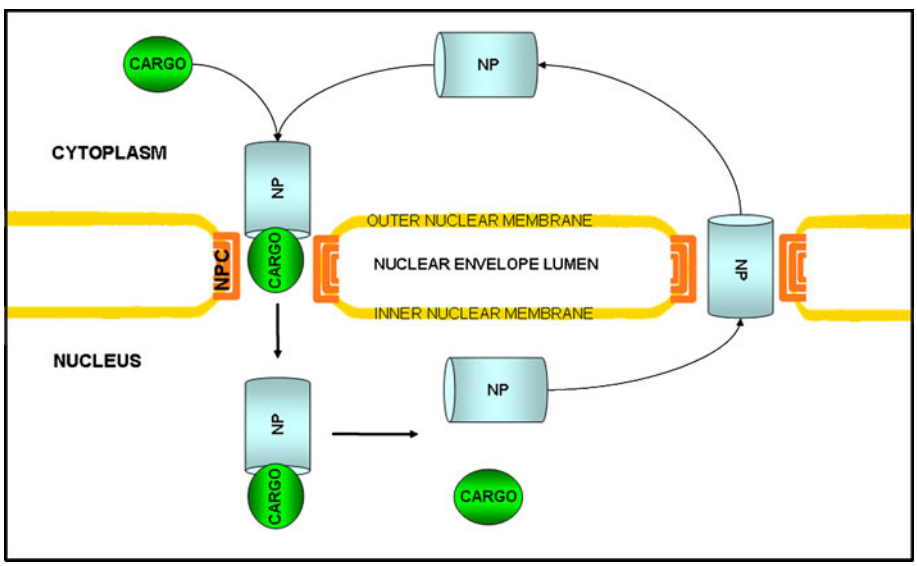

Fig. 2 Scheme showing the mechanisms of nanoparticle (NP) crossing through the nuclear pore complex (NPC) to deliver its attached cargo.

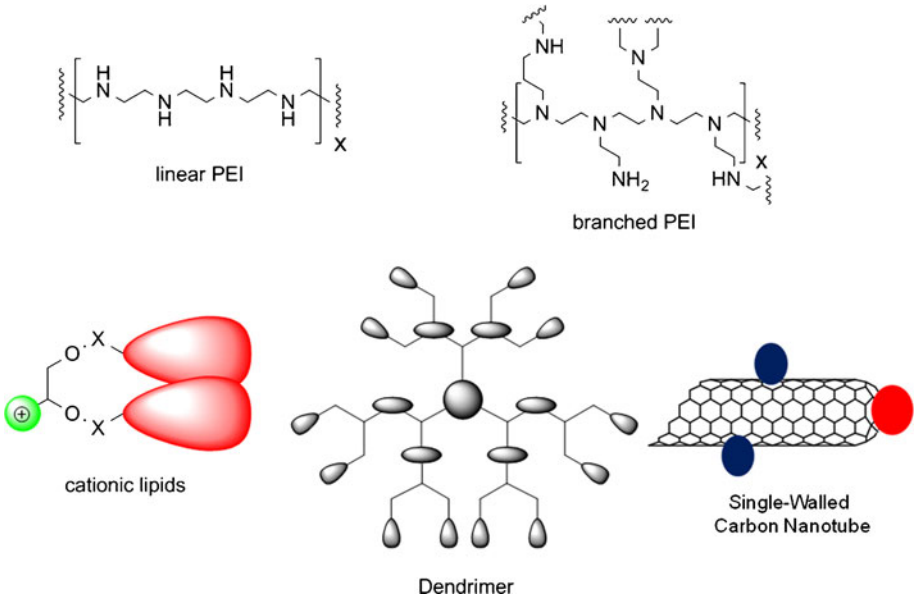

Fig. 3 Structure of different nanoparticles. Linear polyethyleneimine, branched polyethyleneimine, cationic lipids where the *green ball* represents the cationic polar head group, while the red tails symbolizes the hydrophobic carbon skeleton, dendrimer and a single-walled carbon nanotube where the *blue* and *red balls* represent different functionalizations.

Lipofectamine Plus resulted in the presence of rhodamine-labeled DNAs in the nucleus of the cells within 0.5 to 1 h of incubation, thereby indicating that DNA can enter the nucleus in the presence of the nuclear membrane (52). The fact that only naked DNA was visualized in the nucleus indicated that the lipoplex-containing DNA fused with the nuclear membrane and released naked DNA into the nucleus (52). The driving force proposed for the lipoplex to gain access to the nucleus from cytoplasm involves electrostatic interactions between the positively charged lipoplex and the negatively charged lipids in the nuclear membrane. Lipoplex fusion with the nuclear membrane might therefore be interrupted by non-specific binding with other membranes of intracellular organelles, such as mitochondria, thus resulting in low levels of aggregates reaching the nucleus.

The fusion hypothesis was also supported by reports showing that lipofection allowed transgene expression in neuronal cells (3). This hypothesis would also explain the low levels of transfection efficiency reported for delivery of pDNA to neuronal cultures, with only Lipofectamine 2000 reaching a transfection efficiency of about 25% in primary cortical and hippocampal neurons (3, 53).

One of the main strategies used to improve pDNA import into the nucleus, especially in non-dividing cells, involves the non-covalent and covalent attachment of NLS peptides or nuclear proteins to the pDNA itself or a cationic counterpart (54). The need for nuclear targeting agents in lipoplex vector design was initially described by Holmes and co-workers after examining the DNA distribution in human airway epithelial cells after lipoplex delivery (55). However, it should be noted that although nuclear targeted proteins facilitate nuclear transport of pDNA, it is unclear how efficiently these proteins release DNA once the complex arrives to the nucleus. In agreement with this, it has also been reported that NLS peptides could result in a loss of biological activity of pDNA (56).

PEI Derivatives (Cationic Polymers)

Polyethyleneimine is an organic molecule with a high cationic-charge-density potential based on the presence of multiple amino groups within its backbone (Fig. 3). In addition, PEI is able to retain an important buffering capacity at virtually any cellular pH, thus protecting pDNA and avoiding lysosomal nuclease degradation.

Once PEI polyplexes escape from the endosome, they enter the nucleus by a mechanism which is still unclear. Some studies have suggested that nuclear entry of the DNA complexed with branched or linear PEI does not require cell division (50), whereas others appear to show a cell-cycle-dependent transfection efficiency for branched PEI/DNA polyplexes (57).

A mitosis-dependent mechanism would be consistent with the low transfection efficiency obtained in postnatal primary neurons, including primary cultures of granular cells of the cerebellum, hippocampal pyramidal neurons, primary sensory neurons of the dorsal root ganglia and sympathetic neurons from the superior cervical ganglia (58). Initial experiments in this area showed that the transfection efficiency was about 1% for cerebellar granule cells and about 20% for sympathetic neurons from the superior cervical ganglia. However, although a mitosis-dependent mechanism has been postulated for branched PEIs, better transfection efficiencies in rat cerebral glial cells and rat cortical neurons have been achieved using low-molecular-weight branched PEI derivatives (59), thus indicating that

even if the main mechanism of nuclear delivery is mitosis-dependent, other mechanisms, probably passing through the NPC or neuronal uptake mechanisms, also contribute to polyplex translocation into the nucleus.

Once in the nucleus, cationic polymers appear to dissociate from pDNA by exchange of the polymer with the components of the surrounding chromatin. Thus, dissociation of the PEI-DNA complexes seems to be the most important factor for transgene expression (60). A recent study has shown that a greater number of pDNAs are delivered into the nucleus by PEI than by Lipofectamine. However, although fewer pDNAs reached the nucleus after transfection with Lipofectamine, a higher percentage of the cells transfected with Lipofectamine expressed the reporter gene (61). This is probably due to differences in the transcriptional activity of pDNAs transfected into cells using either cationic lipids or cationic polymers. Dissociation of pDNA from cationic polymers in the nucleus may interfere with transcription until the polymer completely dissociates from DNA. Moreover, it has been described recently that cationic polymers condense pDNA more efficiently than cationic lipids and that pDNA decondensation in the nucleus, which is required in order to use the innate transcription machinery and to express the encoded protein, appears to be the major limiting step for transgene expression (60).

Since it has been established that cell division is not absolutely required for nuclear entry by polyfection, several strategies have been developed to overcome the nuclear barrier, including NLS, glycosyl residues or glucocorticoid ligands. Recently, it has been described that coupling a classical (SV40) or non-classical (TAT) NLS peptide to PEG-DNA binding peptides-PEI increased transfection efficiency (62).

The use of glucocorticoid receptor (GR) ligands as an NLS to enhance transgenic expression is an alternative strategy. Glucocorticoid receptor is a nuclear receptor which is mainly located in the cytoplasm. Upon binding to the ligand, GR translocates from the cytoplasm into the nucleus, dilating the nuclear pore by up to 60 nm and facilitating the transport of associated molecules. The combination of different glucocorticoids with branched PEI has been found to efficiently condense pDNA into small NPs with sizes of around 100 nm. The transfection efficiency increased in the same order as the potency of the glucocorticoid irrespective of the cell type (63). Moreover, triamcinolone acetonide (TA), a potent glucocorticoid, translocates PEI 600-TA and PEI 1800-TA into the nucleus more efficiently than PEI 25 k-TA (64).

Dendrimers

Dendrimers are highly ordered and well-defined macromolecules that will be described in more detail below (Fig. 3). Biologically, these molecules could efficiently induce expression of a reported gene in a variety of suspension- and adherent-cultured mammalian cells. Dendrimers are the non-viral vectors which display the highest transfection efficiency in neuronal cells, with arginine-grafted G4-PAMAM dendrimer, for example, achieving a transfection efficiency of 35%–40% in primary cortical neurons (65). Likewise, PAMAM esters allow siRNA delivery to a primary culture of mixed cortical cells containing neurons and glia, achieving an 80% reduction in the target protein (66), and the ammonium-terminated carbosilane dendrimer NN16 has been used to efficiently transfect siRNA in a primary culture of rat cortical neurons, with a resulting reduction in the total protein content of about 80% (67).

Different dendrimer modifications have been performed to improve pDNA transfection efficiency. Thus, the combination of G4-PAMAM dendrimer with glucocorticoid dexamethasone (PAM-Dex) facilitates polymer/pDNA complex nuclear translocation and increases pDNA transfection delivery in Neuro2A cells more than two-fold with respect to PEI and more than six-fold with respect to PAMAM alone (68). Similarly, the combination of the highly potent glucocorticoid TA with PAMAM G4 dendrimer resulted in a transfection efficiency more than four times higher than native PAMAM. These results support the hypothesis that TA residues act as an NLS, thereby facilitating PAMAM polymer/pDNA entry into the nucleus and enhancing transgene expression (63).

Although numerous efforts have been focused on increasing nuclear pDNA delivery to enhance transgene expression by modifying the different non-viral vectors available, it should be noted that, in contrast to pDNA delivery, the use of small interference RNA (siRNA) avoids the need for nuclear translocation and greatly improves transfection of post-mitotic cells (69). In this regard, Zou and co-workers have recently described that siRNA is between two and five times more efficient than DNA in transfecting cells in a primary mixed neuronal culture (70).

CROSSING THE BLOOD-BRAIN BARRIER

The following section will focus on a brief description of the BBB and the characteristics common to those species that are able to cross it. Recent reviews have covered this subject in greater detail (71).

The BBB is a physical barrier which limits the brain uptake of the vast majority of neurotherapeutic and neuroimaging contrast agents. This barrier normally has to be overcome to reach brain tissue, although an alternative pathway using the naturally occurring trans-synaptic retrograde transport route of entry into the CNS

from the peripheral nerves could be another possible route for CNS entry. Nanotechnology may provide an effective means of delivering therapeutic agents into the CNS following peripheral administration, although NPs must either first overcome the BBB or circumvent it by traveling through peripheral nerves.

The BBB is composed of a dense layer of capillary endothelial cells facing the bloodstream which separates the CNS (i.e. brain and spinal cord) from the rest of the organism by means of tight junctions (*zonulae occludens*). On the brain side, this layer of non-fenestrated endothelial cells is lined by astrocytes, pericytes and neurons. Paracellular transport in brain capillary endothelial cells is restricted by expression of tight junctional proteins that afford high resistance to the cell, and this is associated with limited endocytosis across the endothelium (72). The blood-brain barrier is known for its high selectivity as well as its high transendothelial electrical resistance, with reported values ranging from 1500 to 8000Ω cm^2 (depending on the authors) (73). This value is about three orders of magnitude higher than that present in other tissues (3–33Ω cm^2), thus resulting in a reduction of the aqueous paracellular diffusion. Indeed, only small hydrophilic compounds with a mass lower than 150 Da and highly hydrophobic compounds with a mass lower than 400–600 Da can cross the membrane by passive diffusion.

Characteristic features of BBB microvessels include their smaller diameter and the fact that their walls are thinner than those of other vessels in the organism. These vessels are highly dense in mitochondria, mainly due to the lack of cytoplasm surface in their endothelial cells. As a result of this high mitochondrial area, those drugs that cross the BBB are very likely to be degraded enzymatically. In terms of selectivity, the efficient efflux pump system composed of *p*-glycoprotein located in the cerebral capillary endothelium should also be mentioned. This pump is involved in recognition of those molecules necessary for the brain and the prevention of other molecules from entering the brain parenchyma (74). Moreover, transcytosis, the process used by nanocarriers to pass across the BBB, is partly prevented by the activity of efflux pumps like p-glycoprotein. NPs should therefore gain access to the CNS by lipid-mediated free diffusion or potentially by receptor-mediated endocytosis.

The distinctive chemical nature of those drug molecules that have been proved effective in the CNS (e.g. hydrophobic benzodiazepines and the highly polar L-DOPA) makes it difficult to achieve a comprehensive understanding of the mechanisms that regulate their passage across the BBB. Moreover, based on the transport of some hydrophilic drugs, several NPs can transiently and reversibly open the tight junctions located at the BBB and other sites, thus increasing their paracellular permeability (75). Nevertheless, it is known that tight junctions can be opened up to 20 nm wide (76); thus, only NPs smaller than this can use this pathway to penetrate into the brain through the BBB.

Trans-synaptic retrograde transport could enable some types of nanocarriers with a specific size and surface modifications to travel from peripheral nerve terminals to neuronal cell bodies in the CNS (as occurs with the transport of some pathogens and toxins), where they can deliver therapeutic agents (77). Studies in this regard have shown that NPs modified with PEI and other polyplexes display active retrograde transport along neurites but are unable to mediate efficient biological actions upon reaching the neuronal body (78). In contrast, other studies have shown that some NPs cannot travel through peripheral nerves, although they produce efficient neuronal transfection if they are delivered directly to neuronal cell bodies (78). It has been proposed that the differences in uptake pathways and efficiencies between neuronal soma and neurites are related to either the differing surface properties of both cellular areas (79), the lower efficiency and higher selectivity shown by neuronal processes for internalizing small size particles (80), or the inability of neuronal processes to internalize and transport relatively large NPs to cell bodies.

Transcellular transport may be mediated by transferrin (Tf) receptors, which are highly expressed in some healthy tissues such as brain capillaries. Interestingly, several *in vitro* and *in vivo* studies have shown that NPs conjugated with Tf penetrate the BBB much more easily, thereby significantly improving the delivery of active molecules to rat brain with respect to the same NPs devoid of the ligand (81).

Other strategies to increase passage through the BBB have been studied. Thus, nanoparticles conjugated with PEG or monoclonal antibodies have been shown to translocate into brain tissue after intravenous administration, thus suggesting a critical role for these proteins in NP endocytosis by the brain endothelial cells (82). Surface modifications of NPs in order to improve their passage across the BBB can, however, decrease their ability to breach other barriers; therefore, the delivery of active molecules by NPs into cells of the CNS remains a challenge.

NANOPARTICLES AND THEIR ROLE IN GENE DELIVERY TO THE NERVOUS SYSTEM

Nanomaterials have at least one of their dimensions within the nanometer (10^{-9} m) range and exhibit electromagnetic, photophysical and/or structural properties that are directly related to their nanometric size. Indeed, the size of these materials has been shown to result in interesting biological properties, some of which have been successfully applied to gene-delivery processes.

The efficiency of the uptake process using non-viral gene-delivery agents depends on the cell type, the target site and the specific carrier composition. In the case of neurons, this is very important since neurons are highly polarized cells whose somal, axonal and dendritic domains have distinct membrane compositions (80).

In this section, we will study the effectiveness of the aforementioned nanoparticles to deliver genetic material (DNA, mRNA or siRNA) to the cytoplasm of CNS cells, taking into account that neurons are a difficult cell type to transfect both *in vitro* and *in vivo*.

Cationic Lipids and Cationic Liposomes

Efforts to find efficient vectors for gene therapy have also focused on small molecules, such as liposomes, which have been found to enter cells through RME via the CCP pathway as well as by direct fusion with the membrane (83, 84). Since the synthesis of DOTMA (*N*-2,3-dioleoyloxy-propyl)-*N*,*N*,*N*-trimethylammonium chloride) by Fegner and co-workers (85), which was subsequently commercialized as part of Lipofectin®, cationic lipids have been widely used in gene transfer (86). Their structure involves a cationic head group, a linker and a hydrophobic part. The positive charge allows interaction with the genetic material by means of electrostatic interactions. Cationic lipids generate liposomes, which are hollow spheres with an aqueous core with a diameter of 100 to 400 nm (85). The surface of the liposome is positively charged and can therefore interact with the negatively charged cell membrane. This results in the DNA becoming coated and the formation of a lipocomplex, which is subsequently incorporated into the cell by endocytosis (87).

In terms of cell differentiation in the CNS, it has also been shown that the binding and internalization of cationic lipid-based gene carriers is more efficient at neuronal soma than at neurites (78). Lipofectin® is a very poor transfectant (0.02%–0.5%) in primary septo-hippocampal neuronal cultures (88), although its transfection efficiency increases to about 25% in primary E18 rat cortical neurons and E18 rat hippocampal neurons (2, 87).

One strategy for increasing the transfection efficiency of cationic lipids involves adding molecules that can facilitate either uptake or endosomal escape. The incorporation of targeting ligands such as Tf or antibodies within these lipoplexes has resulted in efficient nucleic acid delivery to neurons in the CNS by means of systemic administration (89). Transferrin receptor is a ubiquitous membrane receptor that can mediate endocytosis, and several cationic lipoplexes associated with Tf have been shown to provide good results in the delivery of anti-c-Jun siRNAs to neuronal cells in culture. This process resulted in the efficient silencing of c-Jun mRNA and protein and a significant decrease of cell death following glutamate-induced damage or oxygen-glucose deprivation. Tf-lipoplexes have also led to a significant c-Jun knockdown in the mouse hippocampus *in vivo*, and it has also been shown that Tf-lipoplexes promote siRNA delivery and siRNA-mediated protein silencing in the brain with high efficiency and minimum toxicity following stereotactic injection (90).

Although an overall positive charge in the lipoplex favours binding to the cell membrane, such binding has also been reported with neutral immunoliposomes (91). PEGylation not only prevents complex aggregation but also several undesirable effects reported for non-PEGylated complexes, including erythrocyte aggregation and binding of complexes to plasma proteins such IgM, fibronectin and complement C3 (92).

Polyethyleneimine Derivatives

As mentioned above, polyethyleneimine (PEI) is an organic molecule produced by the polymerization of aziridine in which every third atom is an amino nitrogen that can be protonated and can bind nucleic acids (Fig. 3). PEI will spontaneously adhere to and condense DNA to form toroidal complexes that are readily endocytosed by cells. Furthermore, it has been shown that PEI complexes are introduced into the cell by adsorptive endocytosis, with no inhibition of the endocytic uptake of a non-digestible fluid-phase substrate such as fluorescein isothiocyanate (FITC)-dextran. However, it has also been reported that PEI/DNA complexes are effectively endocytosed, probably as a result of interaction with cell-surface molecules such as proteoglycans, more specifically syndecans (93).

Linear or branched polyethyleneimines (lPEI and bPEI, respectively) have been considered to be promising cationic polymers that can act as non-viral vectors for DNA or siRNA delivery since the last decade and are currently considered to be gold standards when studying the gene-transfer efficiency of a new vector. bPEI usually gives better transfection results than lPEI, probably as a result of the size of the final polyplex, as lPEI forms larger particles that could be more difficult to endocytose. Thus, a transfection efficiency of about 9% in primary sympathetic neurons was obtained using 22 K lPEI (94), whereas bPEI (600–800 kDa) gave a transfection efficiency of 15% in rat hypothalamic neurons (2). When compounds with a similar molecular weight are compared, 25 kDa bPEI provides higher transfection efficiencies than 25 kDa lPEI in rat cerebral glial cells and rat cortical neurons. As mentioned previously, bPEI generates smaller complexes with the genetic material (59), thereby facilitating uptake by clathrin-mediated endocytosis, where the typical size limitation is about 200 nm. Particle charge may also influence the uptake mechanism and efficiency in the gene delivery (78).

Receptor-mediated endocytosis is the most selective strategy to reach CNS cells. Thus, conjugation of hydrophobic molecules, such as cholesterol, to PEI enables the formation of WSLP (water-soluble lipopolymers), which go on to form micelles in aqueous solution. This micelle formation could condense the WSLP/DNA complex and facilitate its interaction with, and uptake into, target cells. The presence of cholesterol in these micelles could allow the lipoplex to incorporate into LDL and enter cells by LDL receptor-mediated endocytosis (95, 96).

TrkA or the p75NTR receptors for neuronal growth factors (NGF) have been targeted as entrance portals for PEI polymer-based polyplexes. Thus, Zeng and co-workers added the nerve growth factor loop 4 hairpin structure to the polyplex (97) to form complexes which target cultured cells expressing the TrkA receptor. The same authors also used these vector-driven polyplexes in dorsal root ganglia, which resulted in a decrease in the toxicity caused by non-specific uptake of large amounts of higher-molecular-weight PEI. Transferrin has also been successfully added to PEGylated PEI/DNA polyplexes (98).

Neurotoxin receptors are also important receptors to be studied for access to CNS cells. Tetanus toxin (TeNt), for instance, is a good ligand to imitate, with TTC, the purified, recombinant heavy chain fragment of TeNT, being responsible for TeNT cell binding and retrograde axonal transport (4). Liu and co-workers have identified a 12-amino acid peptide, Tet1, which mimics the receptor-binding properties of TTC (99). Conjugation of Tet1 to PEI resulted in specific uptake of Tet1-modified PEI/DNA polyplexes by cells expressing TTC receptors (100).

Dendrimers

Dendrimers are attractive molecules which are related to hyperbranched polymers but with a well-defined structure and a polydispersity close to 1.0. These macromolecules are globular and nanoscaled and have a unique architecture containing three distinct domains: a central core (either a single atom or a group having at least two identical chemical functionalities); branches emanating from the core, which are composed of repeat units with at least one branching junction whose repetition is organized in a geometric progression that results in a series of radially concentric layers named generations (G); and terminal functional groups, which are located on the exterior of the macromolecule and facilitate interactions with solvents, surfaces or other molecules (Fig. 3).

Poly(amidoamine) (PAMAM) dendrimers, also known as Starbust dendrimers, were synthesized by Tomalia and co-workers in 1985 and are probably the most widely studied (101). The transport of PAMAM dendrimers and their derivatives across the cell membrane follows an endocytic pathway (102). Several factors, including the dendrimer's molecular mass, the functional groups on its periphery (103) and cholesterol levels, are known to modulate this transport, thus suggesting that membrane cholesterol and raft integrity are physiologically relevant for the cellular uptake of dendriplexes (104). Dendrimers have been shown to cross cell barriers at sufficient rates to act as potential carrier/delivery systems (105).

It has also been shown that dendrimer nanocarriers can be used to enhance the transport of propranolol across the membrane of Caco-2 cells. Propranolol is an insoluble drug and a substrate for the p-glycoprotein efflux transporter. When conjugated to G3 PAMAM, propranolol was shown to bypass the efflux system. Dendrimer nanocarriers may therefore also be used to enhance the bioavailability of drugs that are poorly soluble and/or substrates of efflux transporters (102).

Transmission electron microscopic analyses of cells incubated with gold-labelled G3 PAMAM dendrimers confirmed endocytosis-mediated cellular internalisation when these dendrimers were applied to the apical domain of Caco-2 cells. These findings are in agreement with similar previous studies using Caco-2 cell monolayers, which showed a significant decrease of dendrimer uptake in the presence of colchicine (endocytosis inhibitor) and when the temperature was lowered from 37 to 4°C (106). These findings are consistent with transport involving both trans- and paracellular pathways (107).

Enhancement of gene transfer has also been observed when PAMAM structures are modified with less polar moieties such as PEG groups. When PEG is located on the periphery of the dendrimer, non-specific adsorption to the cell membrane and subsequent ingestion by endocytosis rather than an electrostatic interaction can be observed in cationic dendrimers (103). The combination of PEGylation and addition of a ligand with affinity for the brain (lactoferrin) also enhances the gene transfer efficiency. The presence of lactoferrin favours endocytosis via a receptor-mediated mechanism (108).

A more efficient way of increasing transfection efficiency involves coupling either proteins or peptides to dendrimers. Thus, when administered intravenously in the tail vein of mice, transferrin-conjugated PEG-PAMAM and lactoferrin-conjugated PEG-PAMAM efficiently deliver genetic material to brain tissue, although maximal gene expression was detected in the kidney for both vehicles studied (108). Target protein expression (GFP) was detected in cortex, hippocampus, caudate putamen and substantia nigra, with lactoferrin-conjugated PEG-PAMAM being more efficient for dendriplex translocation into the brain across the BBB (108).

Angiopep-2 is one of the peptides derived from the Kunitz domain that possesses a high brain penetration capability (109). Thus, intravenous administration of

surface-modified PEG-PAMAM with angiopep-2 into the tail vein of mice has been found to result in a high accumulation of target gene in brain and spleen (110). Furthermore, in this case, internalization of the dendriplex into brain capillary endothelial cells occurred mainly through CvME and, partly, through macropinocytosis (110). To increase NP entry into neurons, dendrimers were covalently linked to rabies virus glycoprotein peptide (RVG29) (111). Following intravenous injection, an accumulation of the target gene in brain to a similar extent to that observed in the spleen and heart could be observed. Gene expression in the former was much higher in the hippocampus and substantia nigra (112). The mechanism involved in particle internalization seemed to be clathrin- and caveolae-mediated energy-dependent endocytosis.

Arginine-modified PAMAM dendrimers show an enhanced gene-transfer activity in primary cortical neuronal cultures (65), possibly due to the localization of the arginine residues on the surface of PAMAM-Arg/DNA complexes, which presumably facilitates uptake or nuclear localization (113). Thus, an arginine-grafted G4-PAMAM dendrimer has been reported to result in transfection levels of 35%–40% in primary cortical neurons. These transfection levels were significantly higher than those reported previously for Lipofectamine®, 25-kDa bPEI and native PAMAM (65). More recently, it has been reported that biodegradable polycationic PAMAM esters in which arginine is bound to PAMAM-OH functionalized dendrimers allow siRNA delivery to a primary culture of mixed cortical cells containing neurons and glia, thereby resulting in a reduction of about 80% in protein levels 12 h post-transfection, (114). Moreover, a second generation ammonium-terminated carbosilane dendrimer containing 16 positive charges (NN16) has been shown to efficiently transfect siRNA into rat cortical neurons. Thus, at 18 h post-transfection, approximately 85% of neurons contained fluorescein-labelled siRNA/2G-NN16 complex. Analysis of the target protein (hypoxia-inducible factor (HIF)) showed a reduction of about 80% of the total protein content with no toxic effects on the neurons (67).

The cytotoxicity of cationic polymers has been reported to be a function of the interaction between the polymer and the cell membrane and/or of cellular uptake efficiency (106, 113).

Carbon Nanotubes (CNTs)

These materials are a new allotropic form of carbon which consist of seamless graphene sheets rolled concentrically to form capped cylinders mostly composed of carbon hexagons and highly strained regions at the tips, where carbon pentagons are the predominant shape. Depending on the number of graphene layers, single-, double- or multi-walled CNTs (SWNTs, DWNTs and MWNTs, respectively) can be obtained. Thus, whereas SWNTs are composed of a single monolayer of graphene, MWNTs are composed of a concentric arrangement of several nanotubes (Fig. 3). These nanomaterials might play an important role in different fields, including nanomedicine (115, 116).

The use of carbon-based nanomaterials has two main disadvantages: lack of solubility and potential toxicity. Pristine carbon nanomaterials have a poor solubility that can be increased markedly by the incorporation of different pendant units (116, 117). These structural modifications can be performed in a non-covalent fashion by taking advantage of electrostatic, π-π or van der Waals interactions, amongst others, or in a covalent manner that results in a chemical change in the surface structure. PAMAM dendrimers have been anchored to the surface of carbon nanotubes (118), and PAMAM dendron fragments attached to the surface of carbon nanotubes have been shown to be efficient for gene transfer (119). Experiments with mice using CD80siRNA-SWNTs demonstrated an important inhibition of CD80 expression in myeloid immunosuppressive cells (120). Moreover, the injection of SOCS1siRNA-SWNTs resulted in enhanced antigen-presenting function of dendritic cells and therefore suppressed tumor growth (120).

The second issue which must be resolved before CNTs can be used in gene therapy is their possible toxicological effects. CNTs are fiber shaped and therefore might behave in a similar manner to asbestos and other pathogenic fibers, which are toxic as a result of their needle-like shape. On the other hand, CNTs are essentially graphitic and are expected to be biologically biopersistent in the organism, mainly in the lungs. Moreover, it has been reported that intratracheal or pharyngeal instillation of a SWCNT suspension in mice resulted in a persistent accumulation of carbon nanotube aggregates in the lung, followed by the rapid formation of pulmonary granulomatous and fibrotic tissues at the site (121), inflammatory reactions of the terminal and respiratory bronchioles, and, in some animals, mild fibrosis in the alveolar septa (122).

Various studies have suggested endocytosis as the cellular uptake mechanism for CNTs (123), although phagocytosis and diffusion are also claimed to be possible mechanisms. In a recent review, it has been concluded that phagocytosis appears to be the internalization pathway for CNT aggregates (124), bundles, clusters or singly dispersed CNTs 1 μm or more in length, while endocytosis is the internalization mechanism for CNTs forming supramolecular structures, and diffusion is the internalization mechanism for submicron CNTs that do not form supramolecular complexes. In any case, the aggregation or individualization

of carbon nanomaterials can determine the entry mechanism into the cell as well as the excretion pathway. For instance, individualization of these materials seems to lead to an excretion pathway through the kidney (118). However, the most widespread point of view is that the uptake of CNTs by cells involves clathrin-dependent endocytosis (123). Endocytosis is an energy-dependent mechanism which is characterized by its temperature dependence (125). Although the majority of published studies agree on an endocytosis-based model (124), energy-independent cell uptake with CNTs has also been reported (126). Indeed, a quantitative kinetic model to correlate endocytosis rate with NP geometry, which accurately describes experimental data sets studying the cellular uptake of SWNTs in NIH-3T3 cells by measuring the SWNTs' intrinsic photoluminescence, has been published recently (127). This model has been validated for DNA-wrapped single-walled carbon nanotubes with lengths ranging from 130 ± 18 to 660 ± 40 nm. The maximum rate observed for CNTs occurs at a length of 320 nm, with longer CNTs showing a lower entry rate.

As an alternative explanation, it has been proposed that both negatively and positively charged single MWCNTs enter human embryonic kidney epithelial cells (HEK293) by direct penetration, whereas MWCNT bundles enter by endocytosis (128).

FUTURE DIRECTIONS

The routine administration of genetic material to neurons for therapeutic purposes will inevitably involve nanomedicine and, more specifically, therapeutic nanoparticles due to the numerous problems posed by viral vectors. However, the development of therapeutic nanoparticles for treating CNS diseases needs to overcome mainly two types of problems before it can be used as a routine therapy: biocompatibility/biodistribution and targeted delivery.

Biocompability and Biodistribution

The most relevant problem is to design nanoparticles able to efficiently cross the BBB. This is perhaps the most important brake to the development of nanoparticles. At the present moment, the most relevant advances came from the use of molecules, like transferrin, which facilitate BBB crossing. During the next few years, we are likely to see the progressive development of new chemical formulations to overcome the BBB problem, thereby allowing a more efficient delivery of nanoparticles to brain tissue. One possible line of research might come from the use of NPs coupled to inhibitors of the efflux pumps. However, a better understanding of the mechanisms involved in NPs crossing the BBB is required for proper design of more effective NPs.

Target-Directed Nanoparticles

The specific delivery of therapeutic nanoparticles is probably the most challenging task which lies ahead in the field of nanoparticle therapeutics. Nanoparticles can be directed to either specific cell types or intracellular organelles.

For cell-type-specific targeting, future developments probably will rely on the use of specific drugs with affinity for different types of receptors that would take advantage of the receptor-mediated endocytotic pathway. An alternative approach might be the development of antibodies against specific membrane proteins in the different types of neurons. Advances in this field are especially relevant for the CNS, where there is little information on what proteins are specific and unique to the different types of neurons and glial cells involved in the genesis of different diseases, thus making it difficult to selectively direct a therapeutic nanoparticle to a given neuronal type. If this problem can be solved, the possibilities of gene therapy in the nervous system will be markedly improved.

The second target-directed approach is to deliver specifically the nanoparticles to different intracellular organelles. For this, it is expected that the coupling of signalling peptides to the different nanoparticles can direct the nanoparticles to the different subcellular organelles. Since different organelle-specific sequences are already known, a rapid pace progress in this field would be expected.

We are now at the beginning of a new area of nervous system therapeutics based on the use of nanoparticles as carriers to deliver genetic material, peptides and drugs to the nervous system. Whether this new area will progress at a fast pace will depend mainly on the solutions that research will provide to the problems raised above.

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REFERENCES

1. Liu XX, Rocchi P, Qu FQ, Zheng SQ, Liang ZC, Gleave M, *et al.* PAMAM dendrimers mediate siRNA delivery to target Hsp27 and produce potent antiproliferative effects on prostate cancer cells. ChemMedChem. 2009;4(8):1302–10.
2. Guerra-Crespo M, Charli JL, Rosales-Garcia VH, Pedraza-Alva G, Perez-Martinez L. Polyethylenimine improves the transfection efficiency of primary cultures of post-mitotic rat fetal hypothalamic neurons. J Neurosci Methods. 2003;127(2):179–92.
3. Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, *et al.* Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods. 2004;33(2):95–103.
4. Bergen JM, Park IK, Horner PJ, Pun SH. Nonviral approaches for neuronal delivery of nucleic acids. Pharm Res. 2008;25 (5):983–98.
5. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci. 2007;10(11):1387–94.
6. Ren Y, Savill J. Apoptosis: the importance of being eaten. Cell Death Differ. 1998;5(7):563–8.
7. Devine DV, Wong K, Serrano K, Chonn A, Cullis PR. Liposome-complement interactions in rat serum: implications for liposome survival studies. Biochim Biophys Acta. 1994;1191 (1):43–51.
8. Gregoriadis G. Liposomes in the therapy of lysosomal storage diseases. Nature. 1978;275(5682):695–6.
9. Herve F, Ghinea N, Scherrmann JM. CNS delivery via adsorptive transcytosis. AAPS J. 2008;10(3):455–72.
10. Pardridge WM, Triguero D, Buciak J, Yang J. Evaluation of cationized rat albumin as a potential blood-brain barrier drug transport vector. J Pharmacol Exp Ther. 1990;255(2):893–9.
11. Mousavi SA, Malerod L, Berg T, Kjeken R. Clathrin-dependent endocytosis. Biochem J. 2004;377(Pt 1):1–16.
12. Marsh M, Helenius A. Virus entry: open sesame. Cell. 2006;124 (4):729–40.
13. Wattiaux R, Laurent N, Wattiaux-De CS, Jadot M. Endosomes, lysosomes: their implication in gene transfer. Adv Drug Deliv Rev. 2000;41(2):201–8.
14. Ogris M, Wagner E. Tumor-targeted gene transfer with DNA polyplexes. Somat Cell Mol Genet. 2002;27(1–6):85–95.
15. Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev. 2007;59(8):748–58.
16. Kirkham M, Parton RG. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. Biochim Biophys Acta. 2005;1746(3):349–63.
17. Jockusch WJ, Praefcke GJ, McMahon HT, Lagnado L. Clathrin-dependent and clathrin-independent retrieval of synaptic vesicles in retinal bipolar cells. Neuron. 2005;46(6):869–78.
18. Miller TM, Heuser JE. Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J Cell Biol. 1984;98 (2):685–98.
19. Evans GJ, Cousin MA. Activity-dependent control of slow synaptic vesicle endocytosis by cyclin-dependent kinase 5. J Neurosci. 2007;27(2):401–11.
20. Clayton EL, Evans GJ, Cousin MA. Bulk synaptic vesicle endocytosis is rapidly triggered during strong stimulation. J Neurosci. 2008;28(26):6627–32.
21. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol. 2007;8(8):603–12.
22. Oh P, Borgstrom P, Witkiewicz H, Li Y, Borgstrom BJ, Chrastina A, *et al.* Live dynamic imaging of caveolae pumping targeted antibody rapidly and specifically across endothelium in the lung. Nat Biotechnol. 2007;25(3):327–37.
23. Jallouli Y, Paillard A, Chang J, Sevin E, Betbeder D. Influence of surface charge and inner composition of porous nanoparticles to cross blood-brain barrier *in vitro*. Int J Pharm. 2007;344:103–9.
24. Wu C, Butz S, Ying Y, Anderson RG. Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. J Biol Chem. 1997;272(6):3554–9.
25. Bruckner K, Pablo LJ, Scheiffele P, Herb A, Seeburg PH, Klein R. EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. Neuron. 1999;22(3):511–24.
26. Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, *et al.* Differential targeting of Shaker-like potassium channels to lipid rafts. J Biol Chem. 2000;275 (11):7443–6.
27. Fernandez M, Segura MF, Sole C, Colino A, Comella JX, Ceña V. Lifeguard/neuronal membrane protein 35 regulates Fas ligand-mediated apoptosis in neurons via microdomain recruitment. J Neurochem. 2007;103(1):190–203.
28. Kokubo H, Helms JB, Ohno-Iwashita Y, Shimada Y, Horikoshi Y, Yamaguchi H. Ultrastructural localization of flotillin-1 to cholesterol-rich membrane microdomains, rafts, in rat brain tissue. Brain Res. 2003;965(1–2):83–90.
29. Swanson JA, Watts C. Macropinocytosis. Trends Cell Biol. 1995;5(11):424–8.
30. Mukherjee S, Ghosh RN, Maxfield FR. Endocytosis. Physiol Rev. 1997;77(3):759–803.
31. Harush-Frenkel O, Rozentur E, Benita S, Altschuler Y. Surface charge of nanoparticles determines their endocytic and transcytotic pathway in polarized MDCK cells. Biomacromolecules. 2008;9(2):435–43.
32. Binder H, Lindblom G. Charge-dependent translocation of the Trojan peptide penetratin across lipid membranes. Biophys J. 2003;85(2):982–95.
33. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. J Biol Chem. 1996;271(30):18188–93.
34. Swanson JA, Baer SC. Phagocytosis by zippers and triggers. Trends Cell Biol. 1995;5(3):89–93.
35. Suk JS, Suh J, Choy K, Lai SK, Fu J, Hanes J. Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. Biomaterials. 2006;27 (29):5143–50.
36. Lakadamyali M, Rust MJ, Zhuang X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell. 2006;124(5):997–1009.
37. Creusat G, Rinaldi AS, Weiss E, Elbaghdadi R, Remy JS, Mulherkar R, *et al.* Proton sponge trick for pH-sensitive disassembly of polyethylenimine-based siRNA delivery systems. Bioconjug Chem. 2010;21(5):994–1002.
38. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci USA. 1995;92(16):7297–301.
39. Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2007;59(8):718–28.
40. Guo S, Huang F, Guo P. Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. Gene Ther. 2006;13(10):814–20.
41. Kale AA, Torchilin VP. Enhanced transfection of tumor cells *in vivo* using "Smart" pH-sensitive TAT-modified pegylated liposomes. J Drug Target. 2007;15(7–8):538–45.
42. Andreu A, Fairweather N, Miller AD. Clostridium neurotoxin fragments as potential targeting moieties for liposomal gene delivery to the CNS. Chembiochem. 2008;9(2):219–31.

43. El-Sayed A, Khalil IA, Kogure K, Futaki S, Harashima H. Octaarginine- and octalysine-modified nanoparticles have different modes of endosomal escape. J Biol Chem. 2008;283 (34):23450–61.
44. Hoen DR, Park KC, Elrouby N, Yu Z, Mohabir N, Cowan RK, *et al.* Transposon-mediated expansion and diversification of a family of ULP-like genes. Mol Biol Evol. 2006;23(6):1254–68.
45. Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J Biol Chem. 1998;273(13):7507–11.
46. Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-viral gene transfer. Adv Drug Deliv Rev. 2005;57(5):755–67.
47. Akita H, Ito R, Kamiya H, Kogure K, Harashima H. Cell cycle dependent transcription, a determinant factor of heterogeneity in cationic lipid-mediated transgene expression. J Gene Med. 2007;9(3):197–207.
48. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem. 1995;270(32):18997–9007.
49. Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol. 2000;18(1):33–7.
50. Brunner S, Furtbauer E, Sauer T, Kursa M, Wagner E. Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation. Mol Ther. 2002;5(1):80–6.
51. Zhou R, Geiger RC, Dean DA. Intracellular trafficking of nucleic acids. Expert Opin Drug Deliv. 2004;1(1):127–40.
52. Kamiya H, Fujimura Y, Matsuoka I, Harashima H. Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. Biochem Biophys Res Commun. 2002;298(4):591–7.
53. Kaech S, Kim JB, Cariola M, Ralston E. Improved lipid-mediated gene transfer into primary cultures of hippocampal neurons. Brain Res Mol Brain Res. 1996;35(1–2):344–8.
54. Branden LJ, Mohamed AJ, Smith CI. A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. Nat Biotechnol. 1999;17(8):784–7.
55. Holmes AR, Dohrman AF, Ellison AR, Goncz KK, Gruenert DC. Intracellular compartmentalization of DNA fragments in cultured airway epithelial cells mediated by cationic lipids. Pharm Res. 1999;16(7):1020–5.
56. Zanta MA, Belguise-Valladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc Natl Acad Sci USA. 1999;96(1):91–6.
57. Grosse S, Thevenot G, Monsigny M, Fajac I. Which mechanism for nuclear import of plasmid DNA complexed with polyethylenimine derivatives? J Gene Med. 2006;8(7):845–51.
58. Lambert RC, Maulet Y, Dupont JL, Mykita S, Craig P, Volsen S, *et al.* Polyethylenimine-mediated DNA transfection of peripheral and central neurons in primary culture: probing Ca2+ channel structure and function with antisense oligonucleotides. Mol Cell Neurosci. 1996;7(3):239–46.
59. Zhang C, Yadava P, Hughes J. Polyethylenimine strategies for plasmid delivery to brain-derived cells. Methods. 2004;33(2):144–50.
60. Matsumoto Y, Itaka K, Yamasoba T, Kataoka K. Intranuclear fluorescence resonance energy transfer analysis of plasmid DNA decondensation from nonviral gene carriers. J Gene Med. 2009;11(7):615–23.
61. Glover DJ, Leyton DL, Moseley GW, Jans DA. The efficiency of nuclear plasmid DNA delivery is a critical determinant of transgene expression at the single cell level. J Gene Med. 2010;12(1):77–85.
62. Moore A, Medarova Z. Imaging of siRNA delivery and silencing. Methods Mol Biol. 2009;487:93–110.
63. Ma K, Hu M, Qi Y, Qiu L, Jin Y, Yu J, *et al.* Structure-transfection activity relationships with glucocorticoid-polyethylenimine conjugate nuclear gene delivery systems. Biomaterials. 2009;30(22):3780–9.
64. Ma K, Hu M, Xie M, Shen H, Qiu L, Fan W, *et al.* Investigation of polyethylenimine-grafted-triamcinolone acetonide as nucleus-targeting gene delivery systems. J Gene Med. 2010;12(8):669–80.
65. Kim JB, Choi JS, Nam K, Lee M, Park JS, Lee JK. Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. J Control Release. 2006;114(1):110–7.
66. Kim ID, Lim CM, Kim JB, Nam HY, Nam K, Kim SW, *et al.* Neuroprotection by biodegradable PAMAM ester (e-PAM-R)-mediated HMGB1 siRNA delivery in primary cortical cultures and in the postischemic brain. J Control Release. 2010;142(3):422–30.
67. Posadas I, Lopez-Hernandez B, Clemente MI, Jimenez JL, Ortega P, de la Mata J, *et al.* Highly efficient transfection of rat cortical neurons using carbosilane dendrimers unveils a neuroprotective role for HIF-1alpha in early chemical hypoxia-mediated neurotoxicity. Pharm Res. 2009;26(5):1181–91.
68. Choi JS, Ko KS, Park JS, Kim YH, Kim SW, Lee M. Dexamethasone conjugated poly(amidoamine) dendrimer as a gene carrier for efficient nuclear translocation. Int J Pharm. 2006;320(1–2):171–8.
69. Posadas I, Guerra FJ, Ceña V. Nonviral vectors for the delivery of small interfering RNAs to the CNS. Nanomedicine (Lond). 2010;5:1219–36.
70. Zou S, Scarfo K, Nantz MH, Hecker JG. Lipid-mediated delivery of RNA is more efficient than delivery of DNA in non-dividing cells. Int J Pharm. 2010;389(1–2):232–43.
71. Yang H. Nanoparticle-mediated brain-specific drug delivery, imaging, and diagnosis. Pharm Res. 2010;27(9):1759–71.
72. Faraji AH, Wipf P. Nanoparticles in cellular drug delivery. Bioorg Med Chem. 2009;17(8):2950–62.
73. Smith QR, Rapoport SI. Cerebrovascular permeability coefficients to sodium, potassium, and chloride. J Neurochem. 1986;46(6):1732–42.
74. Misra A, Ganesh S, Shahiwala A, Shah SP. Drug delivery to the central nervous system: a review. J Pharm Pharm Sci. 2003;6 (2):252–73.
75. Kotze AF, Luessen HL, de Leeuw BJ, de Boer AG, Verhoef JC, Junginger HE. Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). J Control Release. 1998;51 (1):35–46.
76. Adamson RH, Lenz JF, Zhang X, Adamson GN, Weinbaum S, Curry FE. Oncotic pressures opposing filtration across non-fenestrated rat microvessels. J Physiol. 2004;557(Pt 3):889–907.
77. Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, *et al.* VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature. 2004;429(6990):413–7.
78. Bergen JM, Pun SH. Analysis of the intracellular barriers encountered by nonviral gene carriers in a model of spatially controlled delivery to neurons. J Gene Med. 2008;10(2):187–97.
79. Margolis RK, Salton SR, Margolis RU. Effects of nerve growth factor-induced differentiation on the heparan sulfate of PC12 pheochromocytoma cells and comparison with developing brain. Arch Biochem Biophys. 1987;257(1):107–14.
80. Parton RG, Simons K, Dotti CG. Axonal and dendritic endocytic pathways in cultured neurons. J Cell Biol. 1992;119(1):123–37.
81. Lee HJ, Engelhardt B, Lesley J, Bickel U, Pardridge WM. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. J Pharmacol Exp Ther. 2000;292:1048–52.
82. Aktas Y, Yemisci M, Andrieux K, Gursoy RN, Alonso MJ, Fernandez-Megia E, *et al.* Development and brain delivery of

chitosan-PEG nanoparticles functionalized with the monoclonal antibody OX26. Bioconjug Chem. 2005;16(6):1503–11.
83. Park JW, Hong K, Carter P, Asgari H, Guo LY, Keller GA, *et al.* Development of anti-p185HER2 immunoliposomes for cancer therapy. Proc Natl Acad Sci USA. 1995;92(5):1327–31.
84. Park JW, Kirpotin DB, Hong K, Shalaby R, Shao Y, Nielsen UB, *et al.* Tumor targeting using anti-her2 immunoliposomes. J Control Release. 2001;74(1–3):95–113.
85. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, *et al.* Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA. 1987;84 (21):7413–7.
86. Koynova R, Tenchov B. Cationic phospholipids: structure–transfection activity relationships. Soft Matter. 2009;5:3187–200.
87. Ohki EC, Tilkins ML, Ciccarone VC, Price PJ. Improving the transfection efficiency of post-mitotic neurons. J Neurosci Methods. 2001;112:95–9.
88. Yang K, Faustinella F, Xue JJ, Whitson J, Kampfl A, Mu XS, *et al.* Optimizing liposome-mediated gene transfer in primary rat septo-hippocampal cell cultures. Neurosci Lett. 1994;182(2):287–90.
89. Zhang Y, Calon F, Zhu C, Boado RJ, Pardridge WM. Intravenous nonviral gene therapy causes normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism. Hum Gene Ther. 2003;14(1):1–12.
90. Cardoso AL, Costa P, de Almeida LP, Simoes S, Plesnila N, Culmsee C, *et al.* Tf-lipoplex-mediated c-Jun silencing improves neuronal survival following excitotoxic damage *in vivo*. J Control Release. 2010;142(3):392–403.
91. Pardridge WM. Drug and gene targeting to the brain with molecular Trojan horses. Nat Rev Drug Discov. 2002;1(2):131–9.
92. Needham D, McIntosh TJ, Lasic DD. Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. Biochim Biophys Acta. 1992;1108(1):40–8.
93. Mounkes LC, Zhong W, Cipres-Palacin G, Heath TD, Debs RJ. Proteoglycans mediate cationic liposome-DNA complex-based gene delivery *in vitro* and *in vivo*. J Biol Chem. 1998;273:26164–70.
94. Horbinski C, Stachowiak MK, Higgins D, Finnegan SG. Polyethyleneimine-mediated transfection of cultured postmitotic neurons from rat sympathetic ganglia and adult human retina. BMC Neurosci. 2001;2:2. Epub 2001 Feb 16.
95. Kim JM, Lee M, Kim KH, Ha Y, Choi JK, Park SR, *et al.* Gene therapy of neural cell injuries *in vitro* using the hypoxia-inducible GM-CSF expression plasmids and water-soluble lipopolymer (WSLP). J Control Release. 2009;133(1):60–7.
96. Lee M, Han S, Ko KS, Kim SW. Cell type specific and glucose responsive expression of interleukin-4 by using insulin promoter and water soluble lipopolymer. J Control Release. 2001;75(3):421–9.
97. Zeng J, Wang X, Wang S. Self-assembled ternary complexes of plasmid DNA, low molecular weight polyethylenimine and targeting peptide for nonviral gene delivery into neurons. Biomaterials. 2007;28(7):1443–51.
98. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 1999;6(4):595–605.
99. Liu JK, Teng Q, Garrity-Moses M, Federici T, Tanase D, Imperiale MJ, *et al.* A novel peptide defined through phage display for therapeutic protein and vector neuronal targeting. Neurobiol Dis. 2005;19(3):407–18.
100. Park IK, Lasiene J, Chou SH, Horner PJ, Pun SH. Neuron-specific delivery of nucleic acids mediated by Tet1-modified poly (ethylenimine). J Gene Med. 2007;9(8):691–702.
101. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, *et al.* A new class of polymers: starbust-dendritic macromolecules. Polym J. 1985;17:117–32.
102. Jevprasesphant R, Penny J, Attwood D, D'Emanuele A. Transport of dendrimer nanocarriers through epithelial cells via the transcellular route. J Control Release. 2004;97(2):259–67.
103. Kannan S, Kolhe P, Raykova V, Glibatec M, Kannan RM, Lieh-Lai M, *et al.* Dynamics of cellular entry and drug delivery by dendritic polymers into human lung epithelial carcinoma cells. J Biomater Sci Polym Ed. 2004;15(3):311–30.
104. Manunta M, Tan PH, Sagoo P, Kashefi K, George AJ. Gene delivery by dendrimers operates via a cholesterol dependent pathway. Nucleic Acids Res. 2004;32(9):2730–9.
105. Wiwattanapatapee R, Carreno-Gomez B, Malik N, Duncan R. Anionic PAMAM dendrimers rapidly cross adult rat intestine *in vitro*: a potential oral delivery system? Pharm Res. 2000;17(8):991–8.
106. Jevprasesphant R, Penny J, Jalal R, Attwood D, McKeown NB, D'Emanuele A. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. Int J Pharm. 2003;252(1–2):263–6.
107. El Sayed M, Rhodes CA, Ginski M, Ghandehari H. Transport mechanism(s) of poly (amidoamine) dendrimers across Caco-2 cell monolayers. Int J Pharm. 2003;265(1-2):151–7.
108. Huang R, Ke W, Liu Y, Jiang C, Pei Y. The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain. Biomaterials. 2008;29(2):238–46.
109. Demeule M, Currie JC, Bertrand Y, Che C, Nguyen T, Regina A, *et al.* Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector angiopep-2. J Neurochem. 2008;106(4):1534–44.
110. Ke W, Shao K, Huang R, Han L, Liu Y, Li J, *et al.* Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. Biomaterials. 2009;30(36):6976–85.
111. Lafon M. Rabies virus receptors. J Neurovirol. 2005;11(1):82–7.
112. Liu Y, Huang R, Han L, Ke W, Shao K, Ye L, *et al.* Brain-targeting gene delivery and cellular internalization mechanisms for modified rabies virus glycoprotein RVG29 nanoparticles. Biomaterials. 2009;30(25):4195–202.
113. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials. 2003;24(7):1121–31.
114. Kim TI, Bai CZ, Nam K, Park JS. Comparison between arginine conjugated PAMAM dendrimers with structural diversity for gene delivery systems. J Control Release. 2009;136(2):132–9.
115. Kostarelos K, Bianco A, Prato M. Promises, facts and challenges for carbon nanotubes in imaging and therapeutics. Nat Nanotechnol. 2009;4(10):627–33.
116. Wu H-C, Chang X, Liu L, Zhao F, Zhao Y. Chemistry of carbon nanotubes in biomedical applications. J Mater Chem. 2010;20:1036–52.
117. Hirsch A. Functionalization of single-walled carbon nanotubes. Angew Chem Int Ed. 2002;41:1853–9.
118. Lacerda L, Soundararajan A, Singh R, Pastorin G, Al-Jamal KT, Turton J, *et al.* Dynamic imaging of functionalized multi-walled carbon nanotube systemic circulation and urinary excretion. Adv Mater. 2008;20(2):225–30.
119. Herrero MA, Toma FM, Al-Jamal KT, Kostarelos K, Bianco A, Da RT, *et al.* Synthesis and characterization of a carbon nanotube-dendron series for efficient siRNA delivery. J Am Chem Soc. 2009;131(28):9843–8.
120. Yang R, Yang X, Zhang Z, Zhang Y, Wang S, Cai Z, *et al.* Single-walled carbon nanotubes-mediated *in vivo* and *in vitro* delivery of siRNA into antigen-presenting cells. Gene Ther. 2006;13(24):1714–23.
121. Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, *et al.* Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol. 2005;289(5):L698–708.

122. Huczko A, Lange H, Bystrzejewski M, Baranowski P, Grubek-Jaworska H, Nejman P, *et al.* Pulmonary toxicity of 1-D nanocarbon materials. Fuller Nanotub Car N. 2005;13:141–5.
123. Kam NW, Liu Z, Dai H. Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. Angew Chem Int Ed Engl. 2006;45(4):577–81.
124. Raffa V, Ciofani G, Vittorio O, Riggio C, Cuschieri A. Physicochemical properties affecting cellular uptake of carbon nanotubes. Nanomedicine (Lond). 2010;5(1):89–97.
125. Weigel PH, Oka JA. Temperature dependence of endocytosis mediated by the asialoglycoprotein receptor in isolated rat hepatocytes. Evidence for two potentially rate-limiting steps. J Biol Chem. 1981;256(6):2615–7.
126. Kostarelos K, Lacerda L, Pastorin G, Wu W, Wieckowski S, Luangsivilay J, *et al.* Cellular uptake of functionalized carbon nanotubes is independent of functional group and cell type. Nat Nanotechnol. 2007;2(2):108–13.
127. Jin H, Heller DA, Sharma R, Strano MS. Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: single particle tracking and a generic uptake model for nanoparticles. ACS Nano. 2009;3(1):149–58.
128. Mu Q, Broughton DL, Yan B. Endosomal leakage and nuclear translocation of multiwalled carbon nanotubes: developing a model for cell uptake. Nano Lett. 2009;9(12):4370–5.