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The potential for nanoparticle-based drug delivery to the brain: overcoming the blood–brain barrier

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The development of blood–brain barrier (BBB)-targeting technologies is a very active field of research: targeting therapeutic actives to the central nervous system by means of systemic administration means crossing the BBB, and this is now one of the most challenging problems in drug development. The BBB is a unique regulatory system that protects the brain environment by separating it from direct contact with the circulating blood. In doing so, it impedes at the same time the access of a large number of diagnostic and therapeutic agents into the brain parenchyma. One of the possibilities of bypassing this barrier relies on specific properties of nanoparticulate vectors designed to interact with BBB-forming cells at a molecular level, as a result of which the transport of drugs or other molecules (such as nucleic acids, proteins or imaging agents) could be achieved without interfering with the normal function of the brain. This article summarises several recent example applications, presents emerging work and highlights the directions for further developments in this area.

Keywords: blood–brain barrier, drug delivery, drug targeting, nanoparticles

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1. The brain – a challenging site for drug delivery

Brain disorders represent more than one-third of the total burden of diseases in Europe, where about a quarter of the population is affected [1]. There are > 600 disorders characterised by progressive nervous system dysfunction: cerebrovascular (e.g., stroke, brain tumours), neurodegenerative (e.g., Alzheimer's, Huntington's, Parkinson's, Pick's), inflammatory or infectious (e.g., multiple sclerosis, acquired immunodeficiency syndrome, Lyme encephalopathy, herpes encephalitis, Creutzfeld–Jakob, cerebral toxoplasmosis), and these diseases are often associated with the atrophy of the affected central or peripheral structures [2]. Recent data from the World Health Organisation estimate the global burden of neurological disease is extremely high worldwide, and amount to a loss of 14 – 20 disability-free years of life per 1000 person-years, with higher values in low-income countries [3]. Not surprisingly, diseases that tend to affect the elderly (i.e., dementia or Parkinson's) are less common in countries where life expectancy is substantially lower, while other diseases such as stroke and birth hypoxia-ischaemia/trauma more than make up for the difference. It comes as no surprise that the demand for drugs that are effective in the treatment of neurological disorders is expected to grow significantly in the coming years.

Many promising biopharmaceutical agents have been developed, but very few of them (< 5%) can be used to treat the central nervous system, as they cannot get access to the desired site of action in therapeutically relevant quantities

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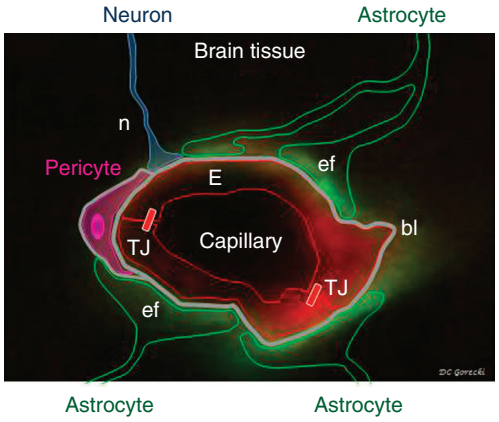

Figure 1. Representation of the constituents of the neurovascular unit involved in the formation and functioning of the blood–brain barrier (BBB). This barrier between the blood and the brain tissue is formed by capillary endothelial cells (E), which are interconnected by means of tight junctions (TJ). The endothelial cells are positioned on the basal lamina (bl) and surrounded by pericytes, with astrocytic endfeet (ef) and neuronal processes (n) closely apposed to the abluminal surface of the capillary. Complex molecular interactions between these elements are essential for establishing BBB, which preserves the brain tissue microenvironment, in turn also preventing drug penetration. The diagram has been superimposed over the fluorescent confocal microscope image of a cross-section through a brain capillary (image courtesy of Dr CF Lien).

because of the blood–brain barrier (BBB) [4]. For the same reason, the vast majority of large-molecule drug candidates (such as monoclonal antibodies, nucleic acids/genes, peptides, recombinant proteins) for central nervous system disorders never make it to the clinic [5]. The BBB has a crucial role in safeguarding the specific microenvironment required for proper brain operation: it ensures the maintenance of the brain interstitial or brain cerebrospinal fluids and the protection of neurons from neurotoxic metabolites, acquired xenobiotics or fluctuations in ionic composition. All these processes must be finely balanced, so neurons can optimally perform their complex integrative functions [6].

Although the BBB relies on the specific properties of brain endothelial cells (BEC) [2], recent studies have indicated that BEC cooperate with various brain cells to generate and maintain the unique barrier properties of the BBB (**Figure 1**). This involves intricate association of BEC, pericytes, astrocytes and neurons into functional 'neurovascular units' regulating the cerebral blood flow and barrier performance. The BBB consists of both structural and functional elements: thanks to significant advancements in recent years, we now have a considerable knowledge on the molecular components of the tight and adherent junctions forming the physical barrier, the enzymes of the metabolic barrier, as well as the diverse transport systems controlling BBB permeability and thus maintaining brain homeostasis [2,7].

The tight junctions are constructed from transmembrane proteins (e.g., occludin, claudins), junctional adhesion and endothelial selective-adhesion molecules. In addition to restricting the paracellular permeability of solutes, tight junctions also help to maintain the polarity of enzymes and receptors on luminal and abluminal membrane domains [8]. The connections between adherens junction proteins (e.g., VE-cadherin, PECAM-1) stabilise further the cell–cell interactions at the junctional zones [2,7,9]. This unique structural and biochemical environment involving complex association of endothelial cells, extracellular matrix, pericytes, neurons, astrocytes, with an ensemble of enzymes, receptors, transporters and efflux pumps is involved in the regulation and control of the delivery of essential nutrients, signalling molecules and other vital materials necessary for the brain. Unfortunately, the same mechanism that protects the brain homeostasis also inhibits the entry of potentially useful diagnostic and therapeutic agents.

Although highly invasive strategies (i.e., intracerebral or intracerebroventricular administration) are useful for local brain delivery in specific cases (e.g., in well-defined tumours), they are risky, costly and of limited value for the administration of therapeutic agents that are directed towards less localised diseases (diffused tumours, Alzheimer's disease, multiple sclerosis etc.) [10].

Numerous intravascular drug delivery strategies, which could result in a widespread transport of the infused drug across the whole brain parenchyma, have been proposed and tested [11]. However, the BBB-imposed challenges to effective intravascular transport still complicate drug delivery and demand the adoption of complementary strategies that promote efficient transport through this barrier; as a result, the last two decades have witnessed many researchers becoming focused on the design of formulations that allow selective transport across the BBB.

2. Physiological transport pathways across the blood–brain barrier

The BBB acts as a physical barrier and regulates the passage of selected molecules between the bloodstream and the brain – by either paracellular or transcellular pathways. The large surface area of the endothelial membrane offers an effective transcellular passive diffusion pathway for lipid-soluble agents and small gaseous molecules, whereas the entry of hydrophilic molecules is generally repressed. Owing to the presence of the tight junctions between the endothelial cells (**Figure 2B**), the passive diffusion of solutes through the paracellular pathway (which is similar to that occurring in epithelia [12]) is also inhibited.

Specific transporters (with activities regulated by the brain's metabolic needs and by the concentration of various substrates in plasma), however, do facilitate the transfer (by means of transcytosis) of many nutrients, including glucose, galactose, amino acids, nucleosides, lactates and pyruvates,

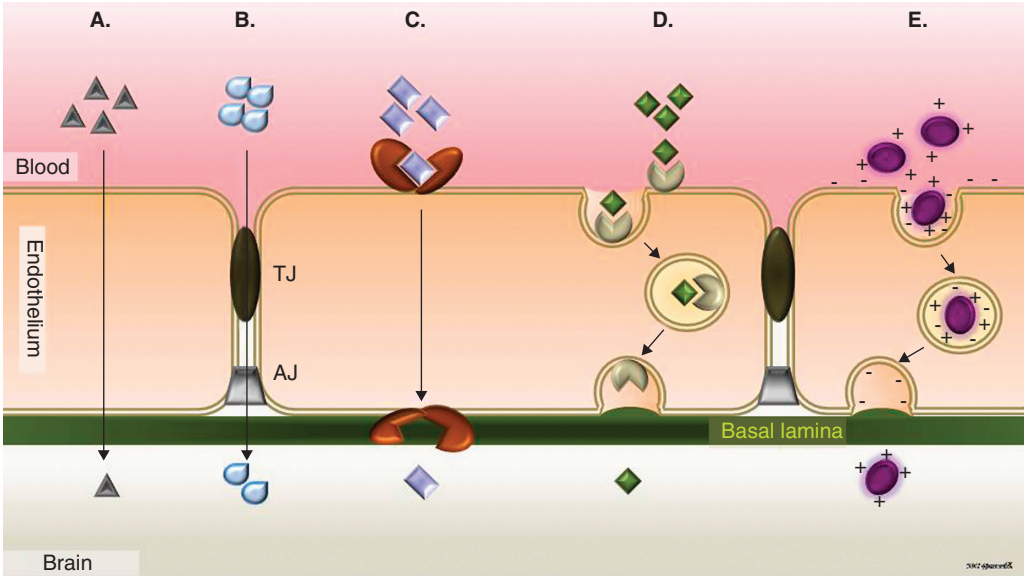

Figure 2. Schematic representation of the physiological pathways involved in molecular traffic across BBB. A. Only lipid-soluble agents can penetrate the endothelial cells. **B.** The tight junctions (TJ) limit penetration of water-soluble compounds, and thus the BBB forces molecules to cross from the blood to the brain compartment through transcellular routes. **C.** Essential molecules (i.e., glucose, amino-acids, nucleosides etc.) are transported with the help of specific carrier/transport proteins. **D.** Specific proteins (e.g., insulin, transferrin) are transported by means of receptor-mediated endocytosis and transcytosis. **E.** Some positively charged molecules can penetrate BBB by means of adsorptive-mediated endocytosis and transcytosis..

adenine and guanine, choline, vitamins and hormones (**Figure 2C**) [13]. As glucose provides the main energy source for the brain, glucose transporters (such as GLUT1 and GLUT3) are of great significance [2]; equally important is the monocarboxylate (lactate; pyruvate) transporter system (MCT1) [14]. Moreover, specialised carriers exist for essential amino acids and vitamins [2].

Some large endogenous proteins and hormones are able to cross the BBB by transcytosis mediated by certain receptors present on the luminal side of the barrier (**Figure 2D**); specific receptors have been identified for insulin, insulin-like growth factors, angiotensin II, folates and transferrin [15]. Polycationic proteins (such as cationised albumins or immunoglobulins) can be transported across the BBB by means of absorptive transcytosis (**Figure 2E**), without the involvement of specific plasma-membrane receptors. In this case, endocytosis is initiated through the association of polycationic substances with the negative charges present on the plasma membrane [16].

At the same time, molecules are continuously eliminated from the brain, and this process is effected by specific active efflux transporters (ATP-binding cassette transporters [ABC]). Each ABC transporter has a different, albeit broad, substrate specificity and presents an effective functional barrier to xenobiotics [17]. In addition, numerous efflux transporters (ASCT2, NET, OAT3) are located at the abluminal side of the endothelial cells membrane, and they all facilitate the efflux of metabolites and neurotoxic compounds that are produced by the brain [14].

These combinations of physical and metabolic protective mechanisms make it extremely difficult to target specific actives into the brain. It is now recognised that for a drug molecule to cross the BBB it must, among other requirements, have high lipid solubility and a molecular mass that is < 400 daltons, and not be a substrate for active efflux transporters [18].

3. Nanoparticles as carriers for drug delivery to the brain

An exciting approach towards the development of a universal 'magic bullet' that can circumvent the BBB and the associated protective mechanisms involves the use of colloidal systems (i.e., micelles, liposomes, nanoparticles) tailored to deliver drugs to the CNS. Although efficient penetration through the BBB and selective targeting are the main requirements of such a drug carrier, ideally it should also achieve long circulation time and external activation or self-regulation of drug release, and have low immunogenicity, good biocompatibility and no clinical side effects. Colloidal drug carriers usually have a size ranging from 1 to 1000 nm and consist of molecular structures within which the therapeutic drugs can be adsorbed, entrapped or attached covalently (**Figure 3**). Such a delivery system may offer increased solubility and enhanced stability to the therapeutic agent (e.g., in case of hydophobic molecules, peptides, oligonucleotides) and may protect it against the many existing natural mechanisms that

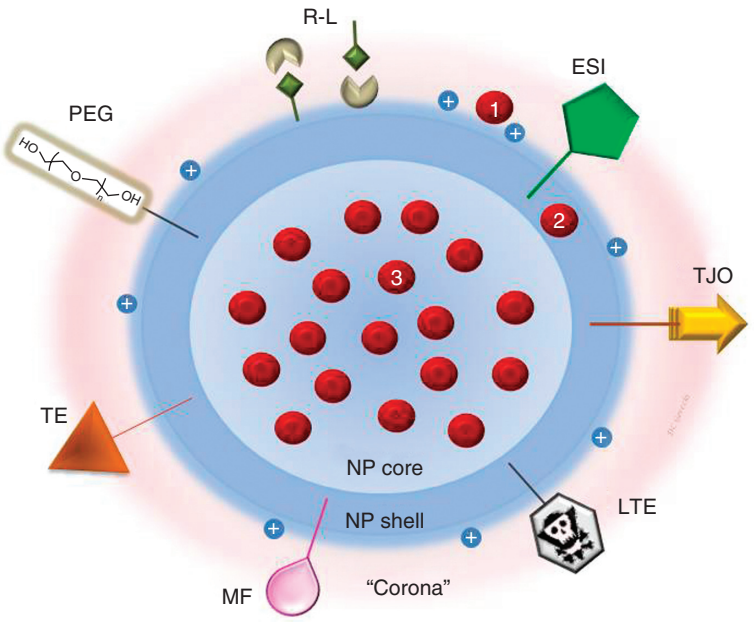

Figure 3. Schematic of a multifunctional model nanoparticle for delivery of actives across BBB. An active (drug, imaging agent) can be adsorbed on the surface (1), enclosed within the NP shell (2), or trapped inside its core (3). The positively charged shell promotes cell surface adsorption and uptake, while pegylation (PEG) increases blood half-life. Different approaches are exploited for targeting: receptor-ligand (R-L) or antibody derivatisation for specific recognition and endocytosis; surfactants (MF) to enhance membrane fluidisation; tight junction openers (TJO) for improved paracellular entry; local toxicity inducers (LTE) to increase permeabilisation of endothelia; efflux systems inhibitors (ESI) to reduce drug efflux; and transcytosis enhancers (TE) to promote nanoparticle transport across membranes. In a biological system nanoparticle surface arrangements are complicated further (and sometimes compromised) by adsorption of a dynamic 'corona' layer of biomacromolecules.

can cause its inactivation and clearance. Owing to preferential accumulation at the target site, the therapeutic agent might also show reduced toxicity.

Although there are still different interpretations of the term 'nanoparticle', it is now generally accepted that it refers to a submicrometre object that 'behaves as a whole unit in terms of its transport and properties' (PAS71, BSI British Standards, UK, 2005). Nanoparticles have their own identity and structural stability, based on covalent bonds or strong ionic interactions. Specific examples of promising carrier systems evaluated recently for brain delivery are presented below. Although other colloidal carriers such as micelles and liposomes also fall within the submicrometre size limit and have been studied extensively for drug delivery to the brain, they possess unique features that distinguish them from nanoparticles, and therefore will not be covered here.

Nanoparticles are versatile platforms for constructing hybrid drug delivery systems with potential for improving pharmacological treatment of brain diseases. The potential mechanism of nanoparticle-mediated drug delivery across the BBB is determined by the chemistry, architecture and properties of the nanoparticles. Several scenarios explaining the observed drug transport are now considered [19]:

- nanoparticles open the tight junctions between endothelial cells and allow the drug to penetrate either in free form or together with the carrier
- nanoparticles are transcytosed through the endothelial cell layer and allow the direct transport of their therapeutic cargo
- nanoparticles are endocytosed by endothelial cells and release the drug inside the cell, as a precursor step to the transport of actives occurring by exocytosis at the abluminal side of the endothelium
- nanoparticles, which combine an increased retention at the brain capillaries with adsorption onto the capillary walls, improve delivery to the brain by creating a concentration gradient that promotes transport across the endothelial cell layer
- drug transport is enhanced by the solubilisation of the endothelial cell membrane lipids by surfactant, which leads to membrane fluidisation (surfactant effect)
- coating agents (such as polysorbates) inhibit the transmembrane efflux sytems (i.e., P-glycoprotein)
- nanoparticles induce local toxic effects at the brain vasculature, which leads to a limited permeabilisation of the brain endothelial cells.

Drugs to be targeted can be incorporated during the preparation stage or adsorbed onto the surface of the pre-prepared nanoparticles. The surface modifications of colloidal delivery systems (e.g., with poly(ethylene glycol)) may bestow prolonged residence in the blood circulation by inhibiting opsonisation and recognition by the mononuclear phagocytic system, and therefore may increase chances of

passive targeting, particularly in microvessels that become leaky in the disease state (e.g., in tumours or inflammatory diseases). Effective, active delivery to the brain may be achievable by coupling the colloidal carrier system with ligands that bind specifically to the receptors located at the BBB. Potential applications and problems associated with intracellular delivery, nanoparticle uptake and trafficking have been reviewed recently by Prokop and Davidson [20]; Govender *et al.* [21], Tosi *et al.* [11] and Jullierat-Jeanneret [22].

3.1 Polymeric nanoparticles

Many of the polymeric particles that can be prepared from natural or synthetic materials possess desirable characteristics of biocompatibility, biodegradability and functionalisation potential that are prerequisite properties of candidate drug carrier materials. Polymeric nanoparticles (NPs) can be prepared from materials such as poly(alkylcyanoacrylates), poly(methylidene malonate), polyesters (poly(lactic acid), poly(lactic-*co*-glycolic acid), poly(ε-caprolactone)), amino acids (poly(aspartic acid), poly(lysine)), polysaccharides (chitosan, alginate), or proteins (gelatine, albumin) [13].

Poly(alkylcyanoacrylate) nanoparticles were developed more than two decades ago taking advantage of the good biocompatibility and *in vivo* degradation potential of this polymer. Such NPs were shown to be able to transport drugs across barriers, allowing delivery of therapeutic doses into difficult-to-reach tissues, including the brain [23]. Polysorbate 80-coated poly(alkylcyanoacrylate) nanospheres have been reported to adsorb apolipoproteins E or B from blood plasma, which indicates that their interactions with specific receptors at the surface of BBB endothelial cells may mimic those of low density lipoproteins [24]. Following the endocytosis of the NP, the drug may be released into endothelial cells and diffuse into the brain parenchyma; alternatively, the particle may be transcytosed. Other processes, such as tight junction modulation or Pgp inhibition, may also occur, and these processes may run in parallel or may act cooperatively to allow efficient drug delivery [25]. Controversially, Olivier has suggested that nonspecific permeabilisation of the BBB may probably be related to the toxicity of the polysorbate 80-functionalised poly(alkylcyanoacrylate) NPs [26]. Resulting studies continue to report increased drug concentration in the brain when polysorbates are used to coat poly(alkylcyanoacrylate) NPs, compared with the uncoated nanoparticles and with the free drug preparations [27,28]. Kreuter and Gelperina reported recently that nanoparticles made of poly(butyl cyanoacrylate) (PBCA) or poly(lactic-*co*-glycolic acid) (PLGA) coated with polysorbate 80 or poloxamer 188 allow the transport of doxorubicin across the BBB, considerably reducing the toxicity of this active [19].

Direct evidence for nanoparticles entering the brain was obtained by using fluorescein-labelled poly(*n*-butylcyanoacrylate) NPs. Although no transcytosis could be observed *in vitro*, the *in vivo* studies showed particle internalisation in the endothelial cells, suggesting passage through the BBB [29]. This emphasises the importance of the *in vitro/in vivo* correlation in nanoparticle studies. Other experiments have shown an evenly spread distribution of fluorescent particles across the brain tissue [30]. In addition to small molecule drugs, poly(butyl cyanoacrylate) nanoparticles have also proved useful for the delivery of plasmids and antisense oligonucleotides to brain tumours [31]. However, there are some concerns about the biodegradation of these materials. Poly(butyl cyanoacrylate)s are prone to hydrolysis and degrade with the formation of water-soluble polycyanoacrylic acid and alcohol [32,33]. The observed cytotoxicity is directly related to the degradation rate, and both appear to decrease with the increasing length of the alkyl pendant chains [26,34]. As a result, the use of alkylcyanoacrylate materials has been approved for external applications (as tissue adhesives) only [35].

Properties such as biocompatibility and 'restorableness' through natural pathways have provided the impetus for the preparation of nanoparticles from poly(lactic acid), poly(glycolic acid) and their copolymers [26]. Degradation of these polymers occurs by means of an autocatalytic hydrolysis of the ester bond into oligomers of lactic and glycolic acid, which ultimately degrade in the Krebs cycle into CO_2 and H_2O [36]. D,L-Poly(lactic acid) nanoparticles surface modified with polysorbate-80 were loaded with nuclear factor-κ B decoy oligonucleotides (ODNs) and used for decoy strategy in the gene therapy of cerebral thrombosis and for the inhibition of tissue factor expression in cultured rat brain microvascular endothelial cells (BMECs). The ODN-containing nanoparticles were not toxic to the BMECs and were localised mostly in the cytoplasm [37]. Poly(lactic acid) nanoparticles loaded with analgesic peptide neurotoxin-I showed improved transport into the brain following intranasal compared with intravenous administration [38]. Breviscapine-loaded poly (D,L-lactic acid) NPs were found to penetrate BBB and enhance the accumulation of actives in the brain [39]. PLA nanoparticles conjugated to TAT cell penetration peptide (see Section 4.2) have been shown to bypass the efflux action of P-glycoprotein, to increase the transport of the encapsulated protease inhibitor across the BBB without disrupting its integrity, and to enhance the CNS bioavailability of the encapsulated active [40].

Biodistribution studies of Tc-99m-labelled chloramphenicol-loaded poly(D,L-lactic-*co*-glycolic acid) nanoparticles administrated intravenously in mice showed relatively high brain uptake and lower accumulation in bone marrow when they were formulated in the presence of polysorbate-80 compared with the formulation in the presence of poly(vinyl alcohol) or with the administration of free drug [41]. Blends of co polymers have also been used to prepare folate-derivatized nanoparticles for targeted paclitaxel chemotherapy. Specifically, poly(lactide)-D-α-tocopheryl polyethylene glycol succinate (PLA-TPGS), which ensures the desired hydrophobic–lipophilic balance, has been combined with TPGS-COOH, which facilitates the folate conjugation [42].

Poly(ethylenimine) (PEI) is a cationic polymer that has shown potential for *in vitro* and *in vivo* delivery of nucleic acids. Mixing cationic PEI with negatively charged plasmid DNA (pDNA) results in the spontaneous electrostatic formation of stable nanoparticle complexes. Recent studies have shown that branched PEI has a stronger electrostatic interaction with pDNA than linear PEI, which accounts for greater compaction, higher zeta potentials and smaller nanoparticle sizes at equivalent pDNA concentrations [43]. Designed changes in molecular architecture of PEI induced by derivatisation with thermoresponsive polymers can be used to enhance gene delivery further [44-47].

Polycefin carriers are built by hierarchic conjugation of functional groups onto the backbone of poly(malic acid), an aliphatic polyester obtained from the microorganism *Physarum polycephalum*. Particular Polycefin variants were shown specifically to target human brain and could be detected easily by non-invasive imaging analysis. Recent studies on the delivery of antisense oligonucleotides against a tumour-specific angiogenic marker using Polycefin resulted in significant inhibition of tumour angiogenesis and increased animal survival [48,49].

Many natural polymers can be fabricated into nanoparticles that are stable in buffered aqueous media and feature a core-shell morphology (**Figure 3**) in the absence of an organic solvent [47,50]. Chitosan is a polymer of natural origin that can be easily formulated into nanoparticles that are stabilised by electrostatic interactions between the positively charged chitosan amino groups and a polyanion such as sodium tripolyphosphate. Such NPs have been used recently for improving nasal absorption and brain targeting of estradiol [51]. Confocal microscope analysis using fluorescent markers demonstrated transcytosis of chitosan nanoparticles coated with the IgG4.1 antiamyloid β-peptide antibody [52] across bovine brain microvascular endothelial cell monolayers [53].

Neutral and cationic ligand-containing maltodextrin-based nanoparticles have been shown to have similar degrees of binding and cell uptake and were subject to transcytosis, indicating that both are good candidate carriers for drug delivery to the brain [54].

Mannan-coated nanoparticles loaded with didanosine showed increased *ex vivo* cellular uptake and higher localisation of didanosine in the brain *in vivo* compared with a drug solution in PBS [55].

Albumin nanoparticles possess surface amino and carboxylic groups that are available for covalent modifications and drug or protein attachment. DNA-albumin is known to avoid opsonisation and uptake by the mononuclear phagocytic system, so albumin nanoparticles have also been investigated as a means for DNA delivery to the brain [24].

Polymer molecules containing cascades of branches grown from a core, known as *dendrimers*, can form small nanoparticles (5 – 20 nm) with very specific architecture, allowing easy attachment of drug molecules or targeting groups [56]. Dendrimers are promising as drug carriers owing to their well-defined size, tailorability and multifunctional nature. Biocompatible dendrimers include poly(amidoamine), poly(propylene imine), as well as several peptide- and polyester-based systems [57]. Non-covalent encapsulation of solutes within the dendritic boxes does not afford control over the release of molecules from the dendrimer, so the covalent attachment of drug molecules onto its periphery is considered a better alternative for drug delivery [50,58]. Poly(ether)-*co*-poly(ester) dendrimers loaded with rhodamine B were able to cross an *in vitro* BBB model, causing only a minor reduction in transendothelial electrical resistance [59]. Some results suggested that the chemical composition and the architecture of dendrimers play a major role not only in influencing the extent and mechanism of uptake by endothelial cells, but also in the permeation across the BBB (i.e., an increase in the number of PEG chains at the surface was found to augment the permeability). Interestingly, the size of dendrimers did not influence the uptake by the endothelial cells. In turn, surface properties are of great importance for the CNS delivery, and toxic cationic dendrimers that are capable of opening the tight junctions of endothelial cells have been shown to have reduced toxicity following surface functionalisation with neutral or anionic moieties [57].

3.2 Solid lipids

Solid lipid nanoparticles (SLNs) within the 100 – 200 nm size range are biocompatible and biodegradable under physiological conditions, have good stability, and can be prepared with excellent reproducibility by means of hot high-pressure homogenisation processes or microemulsification [6]. SLNs exploit the physicochemical characteristics of solid lipid molecules (i.e., triglycerides, fatty acids and waxes) to facilitate the effective delivery of drugs by means of paracellular transport. SLNs can be used as controlled release devices with the therapeutic agent being directly incorporated into the lipid core or associated with the surface of the particles [13]. Compared with polymeric nanoparticles, SLNs have lower cytotoxicity, higher drug loading capacity, absence of burst effect and better production scalability [6,60]. However, the low hydrophilic drug loading capacity of solid lipid nanoparticles limits their drug carrier potential to hydrophobic drugs, proteins and peptides [61-65]. In addition, *in vivo* studies of a solid lipid nanoparticle system formulated with cisplatin revealed that the drug was preferentially targeted to liver followed by brain as a secondary site [66].

Solid lipid nanoparticles (~ 160 nm) showed minimal toxicity and enhanced the cellular accumulation of Rhodamine-123 and [^{3}H]-atazanavir (an HIV protease inhibitor) in the human brain microvessel endothelial cell BBB model as compared with their aqueous solutions [67]. It has been reported that SLNs of < 200 nm have long circulation times and that their plasma absorption is suppressed following surface modification with poly(ethylene glycol), thus facilitating the delivery of lipophilic drugs into the CNS [6,13].

3.3 Carbon and inorganic nanostructures

Nanoparticles made of inorganic materials (i.e., silica, alumina or titania) offer several advantages over polymer-based structures. Their method of preparation is relatively simple and affords good control over size, shape and porosity. Ceramic nanoparticles are often biocompatible and their surface is amenable to modification with functional groups for ligand attachment [68]. These materials have already found application in imaging techniques and are now examined as potential drug delivery vehicles. Porous silicon structures, the most widely investigated materials, are often fabricated in suspensions containing sacrificial nanoscale templates [58]; loading the carrier involves mixing the silica shell with the drug and subsequently drying the mixture to facilitate the coalescence of the drug molecules onto the surface of the silica matrix.

Ultrafine silica nanoparticles functionalised with amino groups have been shown to bind and protect plasmid DNA from enzymatic digestion and to effect cell transfection *in vitro*. It has been reported that silica nanoparticles used for the delivery of genes into the mice brain *in vivo* did not induce any apparent systemic or neurotoxic effects [69]. Organically modified silica (ORMOSIL) nanoparticles were developed as gene carriers and showed good gene transfer efficiency in brain cells *in vivo* [70].

The hollow cage-like architectures and surface characteristics of carbon nanotubes and 'bukyballs' make them candidate materials for use as drug carriers. However, there are concerns about their toxicity, particularly about the capacity of fullerenes to induce lipid peroxidation and to generate free radicals [50]. Toxicology studies have suggested that nanosize aggregates of fullerene molecules can enter cells, and that these structures can cross the BBB. The mechanism by which fullerenes penetrate and/or disrupt cell membranes is still unknown but computer simulations have suggested that these materials are not large enough to cause mechanical damage to the membrane [71].

4. Targeting strategies

Osmotic/biochemical disruption of the BBB or chemical modifications of the active are still the typical strategies for targeting small molecule drugs into the CNS. These methods are rather invasive, risky and limited in their pharmacokinetic effect. Most modern targeting strategies, also used in the case of nanoparticles, rely on engineering the surface by functionalisation or coating with specific, either passive or biologically active, molecules.

4.1 Modulation of efflux transporters

Specific efflux transporters located at the BBB could recognise and transport different molecules (e.g., chemotherapeutics, anti-HIV drugs, opioids) that contain common features with their natural ligands. Much research work is focused on the multi-drug efflux transporter Pgp, a member of the large ABC superfamily of ATP-dependent transporters [17]. The modulation of the Pgp transport function may be achieved by downregulating the Pgp expression at transcriptional or translational levels, by altering membrane targeting after the synthesis of the Pgp protein, or by the chemical inhibition of transporter functions. The development of the first two approaches is still in its infancy, whereas the chemical inhibition of transporter functions has been studied extensively [72]. Recently developed third-generation Pgp inhibitors (e.g., tariquidar, zosuquidar, laniquidar) are reported to be highly specific to Pgp and to have fewer interactions with the co-administrated drugs [73].

The use of Pluronic block copolymers was reported to improve drug delivery of therapeutic agents to the CNS, and maximum activity was observed with Pluronics of intermediate hydrophilic-lipophilic properties [74]. Recent studies indicated that, apart from their inert carrier function (i.e., to extend the nanoparticles' lifetime in the blood by preventing clearance by macrophages), they can enhance drug transport across barriers. Pluronic block copolymers act also as biological response modifiers (i.e., causing various functional cell alterations) [47]. They have the ability to incorporate into membranes, followed by subsequent translocation into the cells, where they affect various cellular functions (such as mitochondrial respiration, ATP synthesis, activity of drug efflux transporters, apoptotic signal transduction, or gene expression). Pluronics have been found to have little effect on the other transporter systems located at the BBB [75].

4.2 Biological targeting – endogeneous transporters and peptide conjugations

Specific transport systems located at the BBB could provide an efficient strategy selectively to target and enhance drug delivery to the CNS, through the conjugation of an endogenous transporter directly to the carrier system [22]. Active substrates have been designed around molecular structures that mimic endogenous nutrients or ligands [73]: for example, mannose liposomes and thiamine- or carnitine-coated nanoparticles have been shown to cross the BBB by means of carrier-mediated transport that uses glucose or choline transporters [36]. Receptor-mediated carriers use both endogenous and chimeric ligands. It must be noted, however, that owing to possible interactions with components of the host biological environments and to potential competition with plasma nutrients, the endogenous features of ligands limit their potential as a means of effecting drug delivery. Receptor-mediated transport into the cell can be achieved by the modification of nanoparticles with targeting moieties such as transferrin or insulin [76], and transferrin and folate receptors have been used for conjugation with liposomes, nanoparticles or nano carrier systems [20]. A three-step targeting procedure based on avidin–biotin technology that uses transferrin as the targeting ligand was used for Paclitaxel-containing biotinylated PEG-PLA nanoparticles, increasing the *in vitro* a*nti*tumoural activity of

paclitaxel when compared with the commercial formulation (Taxol®) and non-targeted nanoparticles [77].

The use of chimeric ligands such as peptidomimetic monoclonal antibodies (Mabs, OX26, Fab) involved in receptor-mediated endocytosis is claimed to offer potential targeting advantages because their ligand binding sites are distinct from the endogenous ligand binding sites [36]. There is evidence that not just peptide and protein drugs can be delivered using nanocarriers, but specific peptides can also be used as targeting coatings able to transport nanoparticles with particular properties. Cationic albumin and peptides such as RMP-7 and MMP-2200 derivatives have been conjugated onto nanoparticles for drug delivery across the BBB; anaesthetic agents such as dalargin, kyotorphin, or neuromuscular blocking agent tubocurarine have also been investigated [78]. In an effort to produce carriers with good circulation-stability properties and that are capable of triggering receptor-mediated transport across the BBB, poly(ethylene glycol)-coated chitosan nanoparticles have been functionalised with OX26 monoclonal antibodies against transferrin receptors [79].

Cell-penetrating peptides (CPP), also known as protein transduction domains or membrane transduction domains (e.g., Antennapedia, TAT), are of high interest owing to their capacity to translocate across biological membranes and to aid the transport of various substrates into and across the cell [80-82]. CPP can be cationic or amphiphatic, and consist of 30 or fewer amino acids. Depending on the size and hydrophilicity of the peptide and on the nature of the attached cargo, translocation may occur by means of membrane transduction or endocytosis [16,20]. However, the exact mechanism of cellular entry for CPP is yet to be elucidated. Importantly, it has been shown recently that TAT-enhanced targeting can take place even when TAT is linked to a payload indirectly by means of two non-covalent bonds [44]. This proof-of-principle result indicates that TAT (and potentially other CPPs) can be used for targeting chimeric vectors and therapeutic nano-devices. The combined use of cell-penetrating peptides and nanotechnology offers tremendous potential in the treatment of brain disorders [78,83]. Recent observations suggest that Antennapedia peptide can increase the membrane-penetrating ability of DNA-loaded nanoparticles prepared from either PEI or polyamidoamine (PAMAM). Such NPs could be used as new non-viral gene vectors [84]. Chlorotoxin was used as a targeting ligand for iron oxide nanoparticles conjugated through a poly(ethylene glycol) linker (to both toxin and methotrexate), and preferential accumulation, prolonged retention and increased cytotoxicity in tumour cells were demonstrated [85].

5. Limitations and prospects

One major factor limiting the use of nanoparticles as drug delivery systems is their fast clearance from the blood circulation: most colloidal drug carriers are rapidly removed from the bloodstream after vascular administration, and accumulate in the liver and/or spleen. The extent of their uptake by the reticuloendothelial system is highly influenced by particle size, surface charges and surface properties. As mentioned previously, addition of poly(ethylene glycol) to nanoparticles enhances stability and slows down the clearance of nanoparticles by the mononuclear phagocytic system. An enhanced antiopsonising effect was reported for high density poly(ethylene glycol) coatings with a molecular mass > 5000 daltons [20]. Other molecules such as lipid-based polymers have also been used to impart resistance to nonspecific protein adsorption and cell adhesion, and to effect steric stabilisation [86].

There is increasing evidence that successful drug delivery by functionalised nanocarriers depends largely on their efficient intra/paracellular transport, a process that is not yet fully understood. Therefore, development of new imaging and diagnostic techniques is very important; at present, our capacity to detect and quantify nanoparticles is limited, especially in complex biological samples. Improved nano-tools could allow a direct visualisation of the transmigration ability of various kinds of biomolecule across the BBB; this would also facilitate the development of new nanoprobes for early diagnosis of various disorders of the brain.

Recent successes are the transport of bioconjugated quantum rods (QRs) across an *in vitro* BBB model by means of a receptor-mediated transport, as well as the use of a QR multiplexing technique to compare simultaneously the transmigration efficiency of different biomolecules across the BBB [87]. Quantum dots are now being investigated as cellular tracking probes for nanocarriers to help understand intracellular transport profiles of functionalised nanoparticles. A new technique involving encapsulation of quantum dots into the core of wheat germ agglutinin-conjugated nanoparticles has been developed recently. The resulting nanoparticles did not show any changes in particle size, zeta potential or binding activity and the loaded probe presented excellent photostability and ability of tracking cellular transport of the functionalised nanocarriers [88]. Although the imaging and diagnostic applications of nanoparticles are not part of this review, it must be mentioned here that recent developments in this field are extremely important. They have direct impact on the possibility to investigate the behaviour of the nanoparticle-based drug carrier systems, both *in vitro* and *in vivo*.

Magnetic nanoparticles have become important tools for imaging tissues affected by various diseases and, whereas first-generation nanoparticles were fairly nonspecific, newer generations are targeted to specific cell types and molecular targets by means of affinity ligands. Commonly, these ligands emerge from phage or small molecule screens, or are based on antibodies or aptamers. Some of the recent biomedical applications of NPs as magnetic resonance imaging (MRI) contrast agents, and potentially as carriers for drug delivery, have been reviewed recently [89,90]. Although some authors

consider the potential role of magnetic nanoparticles as drug carriers, at present it appears to be very limited by their restricted loading capacities.

In terms of toxicity, although reducing the particle size of materials is an efficient and reliable way of improving the bioavailability of a drug delivery system, a potentially harmful effect could be expected in return. The unique properties of nanoparticles present serious problems for risk assessment strategies; the current lack of information regarding toxicity and body distribution of nanoparticles creates concern in both the public and the regulatory community, and standard toxicological methods may be inadequate for addressing these issues. Apart from toxicity and side effects following therapeutic administration, it is also important to consider the impact on workers involved in the manufacturing process as well as on the environment. The diffusion and the persistence in the atmosphere of nanoparticles are very different when compared with larger particles with the same chemical composition. Recent reviews on this topic aim both to inform public regarding health concerns and to raise awareness of nanomaterials' toxicity among scientists and manufacturers [91,93]. The use of advanced analytical techniques, such as functional genomics, proteomics and metabolomics, could provide a global assessment of the biological response and will be important in determining the potential toxicity of nanoparticles [94] At the nanoscale, the potential interaction with tissues and cells and the potential toxicity depend on the composition of the nanoparticle formulation. De Jong and Borm, in their overview of systems for drug delivery [91], highlight the importance of toxicology lessons learned from pulmonary applications. However, it cannot be expected that all aspects of nanoparticle toxicology will be detected this way, thus requiring the development of more specific testing methods. Properties of nanotubes suggest that their toxicity may be close to asbestos and some studies found cutaneous, pulmonary, cardiovascular toxicities and carcinogen-like effects [95]. Nano-alumina has been shown to affect the cerebral vasculature [96], whereas silver nanoparticles can traverse the BBB and move into the brain in particle form; once there they may induce neuronal degeneration and even necrosis [97]. Although TiO_2 nanoparticles can promote β-amyloid fibrillation, a process that has been linked to the aetiology of Alzheimer's disease [98], TiO_2-containing Pt(NH3)(4)Cl-2 has been shown to be biocompatible [99].

Nanoparticle-based delivery strategies under consideration include the effect of electromagnetic fields on the BBB transport of antiretroviral agents, such as saquinavir [100].

6. Expert opinion

As compared with other nanostructures such as liposomes or micelles, polymeric nanoparticles are more stable, simpler to prepare and to scale-up, and offer considerable promise as systems for controlled drug release. In addition to issues associated with development costs, the choice of the polymeric matrix is dictated by biodegradability, loading capacity, immunogenicity and toxicity. The organic nature of most polymeric materials offers the further advantage of allowing conjugation with ligands or with other polymers (before or after particle formation), which in turn affords a significant degree of control over the drug release profile and possibility to impart specificity at the tissue or even cellular levels. In addition, particle surface and internal porosity may be manipulated such that nanoparticles meet specified criteria that influence targeting mechanisms (active or passive) and drug release profiles.

A wide range of materials (including solid lipids and polymers of synthetic or natural origin) may be fabricated into nanoparticles that are stable in buffered aqueous media and can deliver therapeutic agents following systemic administration. Owing to their lack of toxicity, good stability and ease of fabrication, solid–lipid nanoparticles represent an imaginative way of administering drugs into the brain, but insufficient solubility of the target drug in the lipid melt limits their usefulness to the delivery of highly hydrophobic actives.

'Second-generation' nanoparticles involve the introduction of surface coatings, each for a different purpose: poloxamers and poloxamines for stabilisation by means of steric effects; PEG coatings to minimise clearance by macrophages and therefore increase the half-life of circulation in blood; polysorbates to improve BBB permeability; and peptide or other coatings to bestow targeting specificity. Despite recent progress in nanoformulation, many challenges remain, such as the unanswered questions regarding the mechanism of polysorbate-coated, nanoparticle-mediated transport of drugs across the BBB: is drug transport enhanced by the solubilisation of the endothelial cell membrane lipids by the surfactant, which leads to membrane fluidisation, or do surfactants inhibit the efflux transport systems, especially Pgp [19]?

The combination of specific materials and cell-based delivery strategies (e.g., complexation with antibodies, cell-penetrating peptides or cell-specific ligands) may represent another tactic for improved targeting. Nanoconjugates are the emerging generation of drug carriers, as such materials have a multimodular structure that may be modified for the simultaneous improvement of drug loading capacity, passage through biological barriers and active targeting of specific cells. Nanoconjugate carriers have evolved from simple, controlled-release devices to modular, multifunctional constructs capable of intracellular targeting and of delivering synergistically functioning drugs.

Critical for the development of effective nanocarrier systems is the understanding of the biophysical interactions between nanoparticles and cell membranes. This is exemplified by the emergence of the concept of nanoparticle-protein 'corona': a dynamic layer of proteins (and other biomolecules) that adsorbs onto nanoparticle surfaces immediately on contact with living systems, and further impacts the cellular behaviour of the whole construct [101,102]. Lynch and Dawson [101] argue that when cell–nanoparticulate interactions are considered,

the studies should not focus on the nanoaparticle itself but on the structure as a whole (i.e., NP and its 'corona' of associated proteins). Indeed, the complexity of the bionanointeractions between nanoparticles and body fluids demands the modification of current approaches and the consideration of the real 'biological identity' of the drug carrier. As formation of a 'corona' is just one of the surface processes that take place when particulates enter living systems, considerable effort needs to be directed at the understanding of such processes before nanoparticles can be appropriately engineered to achieve a desired medical outcome. Studies are necessary on the effects of protein adsorption and on the complex, nanoscale interactions that occur between nanoparticulates, proteins and cells. Only after the conclusion of these studies will particle surface nano-engineering allow for tailored protein adsorption, and for the control of interactions that will eventually result in 'programmed' cellular behaviour.

Integral to advancements in drug delivery is the development of new or improved tools that can make possible the investigation, both *in vitro* and *in vivo*, of the behaviour of nanoparticle-based drug carrier systems. This is exemplified by the innovations facilitated by the development of quantum dots (fluorescence microscopy) and by the advent of magnetic nanoparticles (MRI imaging and magnetic field applications).

The challenges of nanoparticle design for delivery through the BBB are linked intricately with those associated with the detailed appreciation of specific disease-induced changes in the structure and functionality of this biological barrier. Finally, the growing concerns regarding the security, handling, administration and disposal of nanoparticulate systems must be acknowledged and addressed appropriately.

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