

Chapter 17

Nanomedicines for delivery across the Blood-Brain Barrier

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Abstract

Central nervous system (CNS) disorders affect one in three worldwide and represent a large unmet medical need involving chronic conditions such as Alzheimer's and Parkinson's disease,

stroke, brain tumours, migraine, pain, and mental diseases. CNS drug development is hampered by the restricted drug and biological transport across an anatomical barrier, the blood-brain barrier. Many brain tumours and neurological diseases can greatly benefit from the use of emerging nanotechnologies based on targeted nanomedicines that are able to noninvasively transport highly potent and specific pharmaceuticals across the blood–brain barrier. In this chapter, we will discuss blood-to-brain drug delivery strategies using nanocarriers such as polymeric and lipid-based strategies with a focus on the mechanism of permeation, pharmaceutical, pharmacokinetic/pharmacodynamic and regulatory and clinical aspects of their development. Although it remains unrealistic to expect a magic bullet for brain central nervous system delivery, nanomedicines are the only technologies to date to have shown considerable promise for these patients with chronic and devastating brain diseases.

17.1 The Blood-Brain Barrier (BBB); concept and physiology

Neurological diseases, such as cancers, neurodegenerative conditions, infections, pain and psychiatric disorders, multiple sclerosis, epilepsy are a leading cause of disability, morbidity and mortality affecting nearly 1 in 6 worldwide and 1 in 3 in Europe at some point of their lives (Masserini, 2013). At any time 1.5 billion people worldwide are suffering from some form of central nervous system disorder (WHO, 2006), with this number estimated to reach 2 billion as populations are growing and ageing and the prevalence of major disabling neurological disorders steeply increases with age., if curative treatments fail to emerge. Globally the burden of neurological disorders as measured by the absolute number of DALYs that continue to increase (Collaborators, 2019) and with a global cost only to European healthcare budgets estimated to be €800 billion per year (Gustavsson et al., 2011). Delivery of drugs to the brain is difficult, as the vast majority of therapeutic agents are excluded from the brain by the blood-brain barrier (BBB) (Lalatsa et al., 2014). Poor delivery to the brain has been described as a bottleneck in the development of CNS drugs and is responsible for long drug development times and their high failure rate in clinical trials (Kola & Landis, 2004; Pardridge, 2005).

The BBB, is an anatomical barrier that segregates the brain from the circulatory blood, that was proposed one century ago, following Ehrlich's experimental observation that most peripheral organs were stained following an intravenous injection of a water soluble dye with the exception of the brain and the spinal cord (Ehrlich, 1885). Edwin Goldman's follow-up experiments further confirmed the presence of a barrier as injection of trypan blue directly into the cerebrospinal fluid (CSF) stained all cell types in the brain but failed to penetrate into the periphery (Goldmann, 1913). These experiments theorised a bi-directional barrier formed at the level of the brain capillaries. Brain capillaries have evolved to limit the transport of macromolecules (including plasma proteins (Abbott et al., 2010)) and cells between the circulating blood and the brain to ensure that the brain is protected against circulating toxins or infectious agents. The BBB, a term coined by Lisa Stern in 1921 (Bradbury, 1979), is a unique membranous barrier that tightly segregates the brain from the circulating blood and thus by regulating the constancy of the internal environment of the brain within very precise limits, allows for the neuronal functions of the CNS to optimally take place. Unfortunately, the same mechanisms that protect our brains from exogenous compounds need to be overcome by pharmaceutical scientists to provide targeted treatments for neurological disorders.

Three barrier layers contribute to the separation of the blood and neural tissues: i) a highly specialised endothelial cells (EC) layer comprising the BBB and partitioning the blood and brain interstitial fluid, ii) the blood-cerebrospinal fluid (CSF) barrier with the choroid plexus epithelium which produces the

specialised CSF) into cerebral ventricles, and iii) the arachnoid epithelium separating the blood from the subarachnoid CSF (Abbott et al., 2006; Lalatsa & Butt, 2018).

The CNS consists of blood capillaries that are structurally different from those in other tissues in the periphery. The BBB is formed at the level of the endothelial cells of the cerebral capillaries (Figure 17.1) and is comprised of two plasma membrane in series, which are the luminal and the abluminal membranes of the brain capillary endothelium that are separated by about 0.3 μ m of endothelial cytosol (Pardridge & Boado, 1991). The continuous lipid bilayer posed by the capillary walls prevent the entry of large polar or lipid insoluble molecules into the brain with the exception of gaseous exchange. Anatomically, the endothelial cells of the BBB are distinguished from those in the periphery by increased mitochondrial content (Oldendorf, 1977; Oldendorf et al., 1977), a lack of fenestrations (Fenstermacher et al., 1988), minimal pinocytotic activity (Sedlakova et al., 1999) and the presence of tight junctions (Kniesel & Wolburg, 2000) (Lalatsa, Schatzlein, et al., 2012). The endothelial cells are surrounded by pericytes and astroglial foot processes, forming a continuous anatomical and enzymatic barrier (Lalatsa & Butt, 2018). Around penetrating vessels, there is some distance between endothelial cells and brain tissue (Virchow-Robin space), where perivascular macrophages execute CNS immune functions. The intimate contact between neurons, astrocytes, microglia, pericytes and blood vessels along with the functional interactions and signalling between them form a dynamic functional unit known as the neurovascular unit (Figure 17.1).

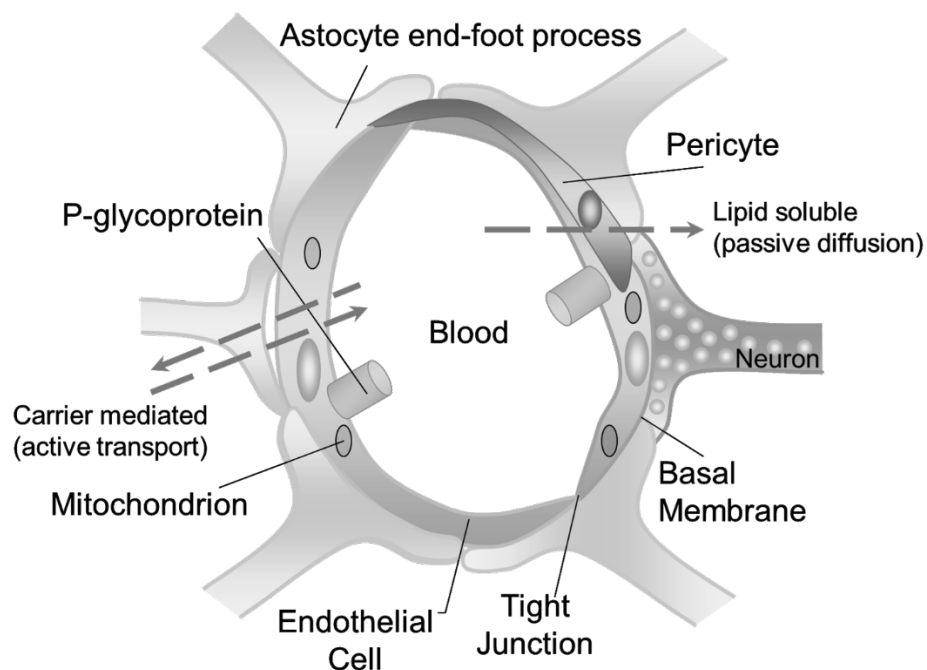


Figure 17.1 Schematic diagram of the neurovascular unit/cell association forming the BBB. Reproduced with permission from (Lalatsa, Schatzlein, et al., 2012).

A single layer of endothelial cells lining the brain capillaries with a higher number and volume of mitochondria forms the innermost luminal constituent of the neurovascular unit. This basement membrane that is 30-40 nm thick is composed of collagen type IV, heparin sulphate proteoglycans, laminin, fibronectin and other extracellular matrix proteins encompass pericytes, endothelial cells and is adjacent to plasma membranes of astrocyte end-feet, enclosing the cerebral capillaries (Hawkins & Davis, 2005). Tight junctions between the capillary cells (zonula occludens) are produced by the interaction of several transmembrane proteins that project into and seal the paracellular pathway resulting in the impermeability of the BBB (Hawkins & Davis, 2005). The interaction of occludin and

claudin and other junctional proteins is complex and effectively blocks the diffusion of polar solutes from blood along these paracellular aqueous pathways denying free access to these solutes into the interstitial fluid (Lalatsa, Schatzlein, et al., 2012). Occludin, a four transmembrane domains protein (60-65 kDa) having the carboxyl and amino terminals oriented to the cytoplasm and two extracellular loops spanning the intercellular cleft of cerebral endothelial cells (Furuse et al., 1993), increases the electrical resistance in tight junctions (TJ) containing tissues mediated by the second extracellular loop domain (Wong & Gumbiner, 1997). The junctional adhesion molecules (JAMs) and the endothelial selective adhesion molecule (ESAM) are members of the immunoglobulin superfamily and are believed to mediate the early attachment of adjacent cell membranes via homophilic interactions (Hawkins & Davis, 2005; Wolburg et al., 2001). Within the cytoplasm are many first-order adaptor proteins, including zonula occludens 1,2, and 3 (ZO) and Ca²⁺ dependent serine protein kinases (CASK), that bind to the intramembrane proteins. Among the second-order adaptor molecules, cingulin is important, and junction-associated coiled-coil protein (JACOP) may also be present (Hawkins & Davis, 2005; Wolburg et al., 2001). Signalling and regulatory proteins include multi-PDZ-protein 1 (MUPP1), the partitioning defensive proteins 3 and 6 (PAR 3/6), MAGI-1–3 (membrane-associated guanylate kinase with inverted orientation of protein–protein interaction domains), ZO-1-associated nucleic acid-binding protein (ZONAB), afadin (AF6), and regulator of G protein signalling 5 (RGS5). All these adaptor and regulatory/signalling proteins control the interaction of the membranous components with the actin/vinculin-based cytoskeleton (Hawkins & Davis, 2005; Wolburg et al., 2001). In epithelial cells, tight and adherens junctions are strictly separated from each other, but in endothelial cells these junctions are intermingled. The most important molecule of the endothelial adherens junctions is vascular endothelial cadherin (VE-cadherin). In addition, the platelet-endothelial cell adhesion molecule (PECAM) mediates homophilic adhesion. The chief linker molecules between adherens junctions and the cytoskeleton are the catenins, with desmoplakin and the p120 catenin (p120ctn) also involved (Lalatsa, Schatzlein, et al., 2012). The endothelium is characterised by exhibiting a high transepithelial electrical resistance in the region of 1500–2000 Ωcm^2 (Butt et al., 1990). Pericytes (granular and filamentous) and endothelial cells are sheathed by the basal lamina that is continuous with the plasma membranes of astrocyte end-foot processes sheathing the cerebral capillaries (Hawkins & Davis, 2005). The ratio of pericytes to endothelial cells is 1 to 3 (Shepro & Morel, 1993). Pericytes seem to play a critical role in the formation and maturation of the BBB during development and regulation of tissue-survival (Daneman et al., 2010), while controlling cerebral blood flow by regulating the capillary diameter through actin fibers in the pericytic cell body (Hamilton et al., 2010). Astrocytes play an important role in maintenance of the BBB, in homeostasis of extracellular concentration of transmitters, metabolites, ions and water. Interaction between astrocytes and neurons determine synaptic transmission, clearance of neurotransmitters, plasticity and blood flow (Wong et al., 2013). The plasma bilayer is high in cholesterol allowing for high packing density of membrane components and therefore an enhanced resistance to passive diffusion of non-cerebral capillaries (Schirmacher et al., 2000). Thus, the BBB apart from preventing the entry of solutes into the brain from the blood, does not allow free diffusional movement of solutes out of the CNS either, hindering free diffusion in a bidirectional way and restricting movement of solutes to transcellular transport (passive diffusion, carrier transport, or endocytotic processes).

Some regions within the CNS lack a BBB, and the capillaries are fenestrated allowing the free movement of solutes between the circulating blood and the surrounding interstitial tissue. These areas around the ventricles of the brain are collectively known as the circumventricular organs (CVO) and comprise of the choroid plexus, the median eminence, the neurohypophysis, the pineal gland, and the organum vasculosum of the lamina terminalis, the subfornical organ, the subcommissural organ and the area postrema. However, the relative surface area of the permeable fenestrated capillaries of the CVO

compared to the tight BBB capillaries is 1 : 5000, making these high permeability areas unable to influence the bulk composition of the brain extracellular fluid and an unrealistic route for drug entry into the brain (Begley, 1996).

The BBB, the choroid plexus epithelium, and the epithelium of the arachnoid matter functions as a physical, transport, metabolic and immunologic barrier able to respond to regulatory signals from both the blood and brain. The barriers are permeable to oxygen and CO₂ and other gases such as helium, xenon, nitrogen and inhalable anaesthetics (Bradbury, 1997). Despite human brain capillaries having an estimated total length of 650 km and a total surface area of 12 m² (Misra et al., 2003), the BBB is very efficient and makes the brain practically inaccessible to polar molecules. Thus, although lipid soluble molecules are able to cross the BBB by diffusion, the endothelial cells are required to maintain a high level of expression of transport proteins for essential polar metabolites such as glucose and amino acids to facilitate their entry into the brain (Begley & Brightmann, 2003).

Apart from being a physical barrier, the BBB is also an enzymatic barrier making drug delivery even more challenging as active agents are exposed to cytosolic and membrane associated enzymes, such as c-glutamyl transpeptidase, alkaline phosphatase, aromatic acid decarboxylase, dipeptidyl(amino)-peptidase IV, and aminopeptidase A and N which are directed at metabolizing neuroactive agents and any agents associating with the barrier (Lalatsa, Schatzlein, et al., 2012).

Additionally, there are a number of efflux transporters located on the luminal membranes of the brain capillary endothelial cells (Begley, 2004a). These ATP binding cassette (ABC) transporters transport lipophilic compounds out of the brain (Terasaki & Hosoya, 1999) against a concentration gradient and among these transporters the P glycoprotein efflux pump (ABCB1) and the breast cancer resistance protein (ABCG2) are important for drug efflux out of the brain while ABCG1 is important for efflux from the cerebrospinal fluid to the blood (Shen et al., 2010; Shen & Zhang, 2010). The BBB is also reinforced in certain disease states such as epilepsy, where there is an overexpression of the P-glycoprotein efflux pump at the BBB (Loscher & Potschka, 2005a, 2005b, 2005c; Volk et al., 2005), while an alteration of the BBB, specifically the activity of the ABC transporters, has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease (ElAli & Hermann, 2011).

17.2 Pathways for transport across the BBB

Despite the impenetrability of the BBB, there are several highly controlled routes across the BBB (Figure 17.2). Polar solutes transport across the paracellular pathway are severely restricted by the presence of tight junctions. However, leukocytes and ions may cross the BBB adjacent to, or by modifying, the tight junctions. Tethering and rolling of leukocytes is achieved via integrins VLA-4 ($\alpha 4\beta 1$) and $\alpha 4\beta 7$ (Laschinger & Engelhardt, 2000) and adhesion molecules such as ICAM-1, VCAM-1 and PECAM-1, contribute to the adhesion and/or migration of distinct subsets of leukocytes to the CNS through cytokine-activated brain endothelium (Greenwood et al., 2003). Lipophilic or amphiphilic solutes may passively diffuse through the large surface of the lipid cell membrane and cross the endothelium. Greater lipid solubility favours this process. Additionally, carrier-mediated influx, which may be passive or secondarily active, can transport many essential polar molecules such as glucose, amino acids, small peptides, amines, monocarboxylates, choline and nucleosides into the CNS. Active efflux carriers may intercept some of these passively penetrating solutes and pump them out of the endothelial cell (e.g. Cyclosporine A, Vinca alkaloids). On the other hand, receptor-mediated transcytosis (RMT) can transport macromolecules such as peptides and proteins across the cerebral endothelium. Examples are transferrin, insulin, leptin, cytokines, viruses (e.g. Herpes Simplex virus). Finally, adsorptive mediated transcytosis (AMT) appears to be induced non-specifically by positively charged macromolecules such as cationised albumin resulting in transport across the BBB. Drug

delivery across the brain endothelium depends on making use of the latter four pathways, while most CNS active agents enter via passive diffusion.

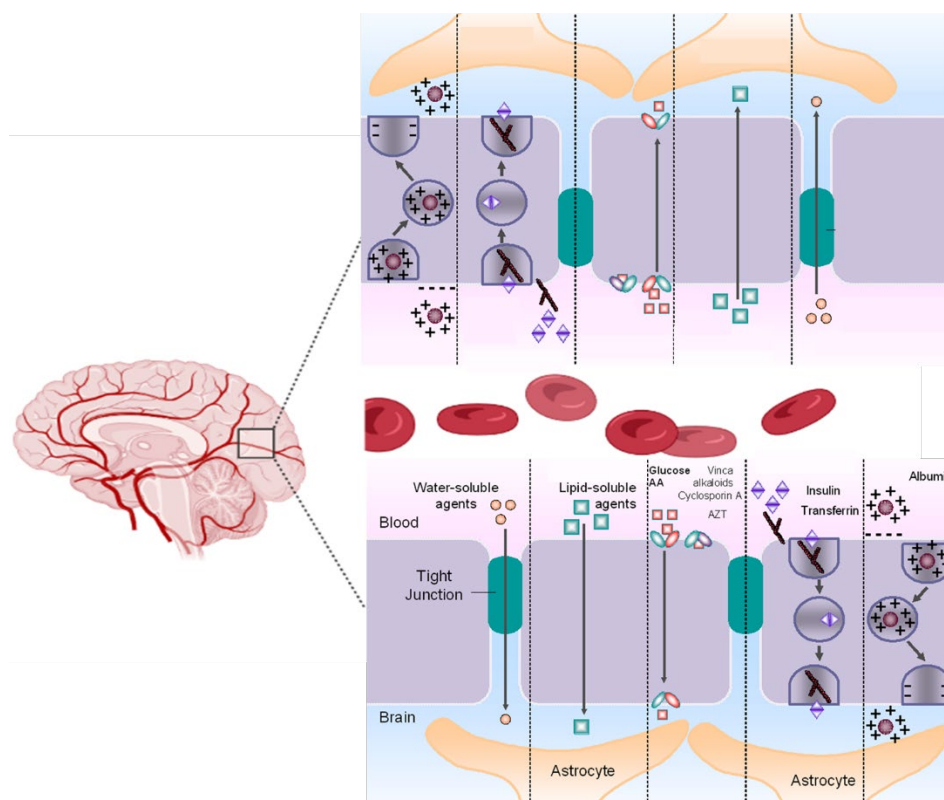


Figure 17.2 Routes for transport across the BBB. Adapted from (Lalatsa & Butt, 2018) using BioRender.com (2022). Retrieved from <https://app.biorender.com/biorender-templates>

17.3 Transcellular permeation of small molecular weight drugs

Drugs with weak hydrogen bonding potential (<6 hydrogen bonds), lipophilicity and small size (<700 Da but typically <300 Da) along with the absence of free rotatable bonds (ideally <8 bonds) (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010) and small polar surface area below 60-70 Å (Kelder, Grootenhuis, Bayada, Delbressine, & Ploemen, 1999) are favourable characteristics for BBB penetration such as in the case of barbiturates and benzodiazepines. Molecular shape is also of importance with spherical size being preferred over rod shape, while there is a negative correlation between branching and ability to cross the BBB (Fong, 2015). However, lipophilic compounds will only accumulate in the brain if they are not a substrate for the ATP Binding Cassette (ABC) efflux transporters (Begley, 2004a). Ionisation state plays a key role in the extent of CNS penetration with basic molecules being generally more CNS penetrant than neutral molecules, followed by zwitterions and finally acidic molecules which are the least CNS penetrant (Gleeson, 2008). Main reason for this, is that bases are positively charged at physiological pH and thus can interact with the negatively charged glycocalyx and phospholipid head groups of the cell membrane facilitating their entry (Gleeson, 2008). Thus an estimated pKa range for BBB permeability is between 4-10 (Fischer et al., 1998) or 7.5-10.5 (Pajouhesh & Lenz, 2005). The presence of positive charge at pH 7-8, or compounds with tertiary amines tend to enhance BBB permeability (Goodwin & Clark, 2005). Strong acids (including carboxylic acids) and bases are generally not easily transported across the BBB (Fong, 2015).

Here it is important to highlight that only the unbound fraction of a therapeutic agent will be available for transport across the BBB. Thus, extensive plasma protein binding (largely binding to albumin) compromises transport across the BBB. Molecular weight and lipophilicity both positively affect plasma protein binding (Gleeson, 2008). High molecular weight drugs are highly protein bound, and

studies have shown that drugs with a molecular weight between 500-700 Da are 98.2% bound (Gleeson, 2008). Ionisation state also plays a role in the level of unbound drug available for transport, as acids are more plasma protein bound followed by neutral compounds, zwitterions and finally bases (Gleeson, 2008). Increasing the lipophilicity of zwitterions and basic compounds, however, can result in an increase in their protein binding that can limit their overall transport (Gleeson, 2008). Brain tissue binding has received less interest than plasma protein binding, however, only the brain free unbound fraction will be available to elicit a pharmacological response. Thus, both the extent of brain permeation and the extent of brain tissue binding need to be considered for a successful CNS delivery.

For all molecules that do not possess these characteristics which is a great variety of drugs and highly specific biologicals agents, transcellular diffusion is not an option and active strategies relying on carrier or receptor-mediated process or adsorptive endocytosis. Few examples exist for drugs and peptides (Lalatsa & Butt, 2018; Lalatsa, Schatzlein, et al., 2012), but for majority of the therapeutics different approaches to overcome the limits posed by the BBB will require a passive or active carrier to get them across the BBB and nanoparticulate technologies offer specific advantages in this that we will explore below.

17.4 Pathways and mechanism of transport of nanoparticulate carriers across the BBB

Several mechanisms for transport of nanoparticulate carriers alone, or loaded or conjugated to therapeutics across the BBB have been exploited in this respect:

17.4.1 Provoking a transient permeability increase in the BBB paracellular pathway.

The disruption of the tight junctions between adjacent endothelial cells is generally achieved by using focused ultrasound and microbubbles (2-5 μ m) after intravenous bolus over 5-10 minutes or continues infusion for multi-target BBB opening (Lapin et al., 2020) and has found significant applications for MRI imaging and other theranostic applications (e.g. increasing the distribution of Herceptin by 50% in glioblastoma multiforme (Kinoshita et al., 2006) and gene delivery) (Chen et al., 2019). Currently, FDA licensed commercialized microbubbles include Optison (GE Healthcare, WI, USA), Definity® (Lantheus Medical Imaging, MA, USA), and SonoVue® (Bracco, Milano, Italy) (Chen et al., 2019). A physical cavitation effect is created from circulating microbubbles, significantly reducing the ultrasound pressure to produce an equivalent acoustic cavitation effect. The subsequent application of ultrasonic energy can achieve a local detachment of tightly sealed junctions on the capillary wall without inducing neuronal damage (Hynynen et al., 2005). Each, however, have different compositions, concentrations, half-lives, and hydrodynamic sizes, which must be considered in terms of impact on interaction between ultrasound-microbubbles and capillary permeability. However, when infusion rates exceeded 20 μ l/kg/min, signs of injury occurred at pressures from 0.39 to 0.56 MPa (Lapin et al., 2020). Additionally, compromising the BBB, can allow circulating toxins or infectious agents to also permeate resulting in toxicities and difficult to treat central infections.

17.4.2 Passive Nanoparticulate Strategies for Brain Delivery.

Passive delivery systems, as opposed to carrier-mediated or receptor-mediated delivery systems that we will explore later, are delivery systems that do not appear to require surface functionalisation so they can bind to carrier proteins or receptors expressed on the surface of the brain capillary endothelial cells. This has been demonstrated for polymeric nanoparticulate carriers based on glycol chitosan amphiphiles (N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan-GCPQ, Molecular Envelope Technology, Nanomerics Ltd (London, UK) (Garrett et al., 2012). These amphiphiles are able to form polymeric aggregates that are able to package and specifically interact with peptides such as Leucine⁵-Enkephalin (an endogenous δ – selective opioid neuropeptide with a plasma half-life of 3 min)

(Lalatsa, Garrett, et al., 2012; Lalatsa, Lee, et al., 2012; Lalatsa, Schatzlein, et al., 2015) and its analogue Dalargin (Mazza et al., 2013) protect them from plasma, liver and brain enzymatic hydrolysis and enable their permeability across the BBB in significant concentrations as evidenced by pharmacokinetic and pharmacodynamic studies. Leucine⁵-Enkephalin MET nanoparticles elicited significant and sustained antinociceptive activity in an acute pain rodent model after both intravenous (Lalatsa, Lee, et al., 2012) and oral administration (Lalatsa, Garrett, et al., 2012).

17.4.3 Carrier-Mediated Transport for Brain Delivery.

Carrier-mediated efflux allows for transport into the CNS of essential polar molecules that cannot diffuse through the cell membrane such as glucose, amino acids and nucleosides (Table 17.1), but exploiting these carrier systems at the BBB can yield successful strategies for delivering drugs and drug loaded nanoparticles to the brain.

Table 17.1. Endogenous transporters controlling the penetration of substrates across the BBB (Lalatsa & Butt, 2018; Lalatsa, Schatzlein, et al., 2012).

Carrier Systems	Abbreviation	BBB location	Orientation	Substrate	Reference
Hexose	GLUT-1	L, A	Blood to Brain	D-Glucose (facilitative, bidirectional)	(Kumagai et al., 1994)
	GLUT-1	L, A	Blood to Brain	Dehydroascorbic acid (facilitative)	(Vera et al., 1993)
Monocarboxylic acids	MCT1	L, A	Blood to Brain	Lactic acid, pyruvic acid	(Kido et al., 2000; Oldendorf, 1973)
Thyroid hormone	MCT8	L, A	Blood to Brain	T3 thyroid hormone (facilitative)	(Friesema et al., 2006; Heuer et al., 2005; Roberts et al., 2008)
Organic anion	OAT1	A	Endothelium to brain	17 β -estradiol-D-17- β -glucuronide	(Sugiyama et al., 2001)
	OAT3	A, L (possibly)	Endothelium to brain	para-aminohippuric acid, probenecid, benzylpenicillin, cimetidine	(Kikuchi et al., 2003; Ohtsuki et al., 2002)
Organic anion transporting polypeptide	OATP1	A	Endothelium to brain	Opioid agonists (N-tyrosinated peptides), pravastatin, glucuronide conjugates, aldosterone, thyroxine, triiodothyronine	(Gao et al., 2000; Gao et al., 1999)
	OATP2	L	Blood to endothelium	Opioid agonists (deltorphin, DPDPE) thyroxine, digoxin, triiodothyronine	(Gao et al., 1999; Kusuhara et al., 1999; Reichel et al., 1999)
	OATP2B1	L	Blood to endothelium	Estrone-3-sulfate (organic anion /bicarbonate exchangers)	(Grube et al., 2006; Nozawa et al., 2004)
Novel organic cation transporter	OCTN2	L, A	Blood to endothelium, Endothelium to brain	Carnitine (organic cation / proton exchange)	(Tamai et al., 1998)
Peptide transport systems 1–5	PTS1-5	L	Blood to brain	Methionine enkephalin, Tyr-MIF-1, TRH, DSIP, α -melanocystimulating hormone, leucine enkephalin (partially)	(Banks & Kastin, 1992, 1997; Ganapathy & Miyauchi, 2005; Maresh et al., 1999)

Glutathione (GSH)	GSH	L	Blood to endothelium	Glutathione	(Kannan et al., 1999)
Amino Acid	Large Neutral (LAT1)	L, A	Blood to brain	Large neutral amino acids: asparagine, glutamate, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, DOPA, cysteine, serine (facilitative, bidirectional)	(Boado et al., 1999; Oldendorf & Szabo, 1976)
	Excitatory (EAAT1, 2, 3)	A	Brain to endothelium	Anionic amino acids, glutamate, aspartate (sodium dependent)	(Oldendorf & Szabo, 1976)
	Cationic (CAT1/ γ + and CAT3)	L	Blood to endothelium	Basic L-amino acids: arginine, lysine, ornithine (sodium independent)	(Smith, 2000)
	Neutral- α	A	Brain to endothelium	Small neutral amino acids: glycine, alanine, asparagine, proline, serine, glutamine (sodium dependent)	(Sanchez del Pino et al., 1992)
	Neutral- β	L, A	Brain to endothelium	Taurine, β -alanine (sodium dependent)	(Komura et al., 1996; Tamai et al., 1995)
	ASCT1, ASCT2	A	Brain to endothelium	L-alanine, serine, cysteine (sodium dependent)	(Zerangue & Kavanaugh, 1996)
Neurotransmitter	GAT	A	Brain to endothelium	GABA (sodium dependent)	(Liu et al., 1993)
	SERT	L, A	Brain to endothelium	Serotonin	(Wakayama et al., 2002)
	NET	A	Brain to endothelium	Norepinephrine	(Wakayama et al., 2002)
Nucleoside	ENT1	L	Blood to endothelium	Thymidine (facilitative, equilibrative)	(Anderson et al., 1999)
	ENT2	L	Blood to endothelium	Adenosine, uridine	(Griffiths et al., 1997)
	CNT1	A	Endothelium to brain	Pyrimidines	(Aymerich et al., 2005; Lu et al., 2004)
	CNT2	A	Endothelium to brain	Purines	(Aymerich et al., 2005; Lu et al., 2004)
	CNT3	A	Endothelium to brain	Pyrimidines and purines	(Aymerich et al., 2005; Lu et al., 2004)
Key: A: Abluminal, L: Luminal					

A well-known example of a drug that utilises carrier proteins for transport across the BBB is levodopa. Levodopa, a lipid insoluble precursor of dopamine, indicated for the treatment of Parkinson's disease, contains the carboxyl and α -amino groups needed for transport across the BBB exploiting the large neutral amino acid carrier (Wade & Katzman, 1975). Chemical groups may be designed and attached to specific pharmacologically active compounds rendering them substrates for these endogenous

carriers, as in the case of Biphalin, an enkephalin analogue (H₂N-Tyr^[1]-D-Ala^[2]-Gly^[3]-Phe^[4]-NH-NH-Phe^[4]-Gly^[3]-D-Ala^[2]-Tyr^[1]), that has been shown to use the neutral amino acid carrier (Thomas et al., 1997). The carrier, that is specific for large neutral amino acids, recognizes a carboxylic acid group and an amino group covalently linked to the same carbon atom, which is a characteristic of an amino group or a conformation that closely resembles this grouping (as in the case of baclophen and gabapentin). A bulky hydrophobic group on the molecule is required to interact with the cell membrane to align the amino and carboxylic groups to the active receptor site, thus excluding amino acids such as glycine and alanine.

Glutathione (GSH) carrier protein has also been used in transport of GSH functionalised particles. GSH tagged pegylated doxorubicin loaded liposomes (2B3-101) compared to pegylated doxorubicin loaded liposomes (Caelyx) resulted in a 4.8 fold higher brain-to-blood ratio without affecting the BBB integrity after a single intravenous dose (7mg/kg) (Birngruber et al., 2014). GSH functionalised pegylated liposomes were also able to delivery antiviral drugs to the brain via the glutathione transporter1 and glutathione coated poly-(lactide-co-glycolide) (PLGA) nanoparticles (NPs) of paclitaxel have been tested for the treatment of brain cancers (Lalatsa, Schatzlein, et al., 2012) (Table 17.2). Recent studies in human have demonstrated their long circulation half-life, linear intravenous pharmacokinetics, good tolerability and ability to elicit near 50% stable disease in breast cancer to brain and high-grade glioma patients (Gaillard, Kerklaan, et al., 2014; Kerklaan et al., 2014).

Some carriers are very selective in their stereochemical substrate requirements such as the Glucose transporter 1 (GLUT-1) carrier, a membrane-spanning glycoprotein containing 12 transmembrane domains with a single N-glycosylation site (Carruthers et al., 2009). Molecules that closely resemble D-glucose (e.g. mannose and galactose (Pardridge, Boado, et al., 1990)) can be transported via this insulin independent glucose transporter expressed in endothelial cells of the brain microvasculature, astrocytes and the choroid plexus. GLUT-1 plays a vital role in brain glucose uptake and hypoglycaemia induces an upregulation of GLUT-1 concentrations (while hyperglycaemia does not seem to exhibit an effect) (Devaskar et al., 1991; Simpson et al., 1999; Simpson et al., 2001). GLUT-1 is not the only glucose transporter at the BBB, but other transporters (e.g. GLUT-4) are also expressed.

Although, many specific transporters have been identified to-date, their exact cellular location at the brain microvasculature and expression level, as well as their selectivity largely determine the kinetics and turnover of the transported nanoparticles or therapeutics (Begley, 2004b; Grabrucker et al., 2016). The hexose and large neutral amino acid carriers have the highest carrying capacity and are the best candidates for delivery of substrates to the brain (Grabrucker et al., 2016). Targeting drugs to a specific nutrient transporter will require a thorough knowledge of both the drug and the transporter for the strategy to yield favourable results. Additionally, dual targeting utilising carrier and receptor-mediated approaches or cell-penetrating peptides (adsorptive mediated approaches) are increasingly utilised (Table 17.2).

Table 17.2 Examples of carrier-mediated transported nanomedicines

Indication	Carrier protein	Nanoparticulate System	Drug/ Therapeutic	<i>In vitro/In vivo</i> findings	Ref.
BCBM or HGG	GSH	GSH decorated pegylated liposomes	Doxorubicin	G-technology (to-BBB technologies BV, Leiden, the Netherlands): 2B3-101 was prepared using a pre-insertion method, in which GSH-PEG micelles were added to the lipids (HSPC and cholesterol) before extrusion and loading of doxorubicin using the ammonium sulphate method at a batch size of 2L. Particles were stable at	(Birngruber et al., 2014; Kerklaan et al., 2014)

				5°C for 18 months. Linear pharmacokinetics after intravenous administration resulting after 3 IV doses of 7mg/kg to double the brain/plasma ratio (>10) at 14 days compared to Doxil in male rats. Phase 1/2 study: 2B3-101 was tolerated up to a dose-intensity of 15 mg/m ² /wk. 2B3-101 showed no neuro-or cardiotoxicity. In 25 evaluable HGG patients, 52% had SD as best response and 3-months PFS rate of 40%. In 23 evaluable BCBM patients, 9% had PR and 48% SD as overall best response with 3-months PFS rate of 48%. AEs ≥ grade 2 were: neutropenia (41%), palmar-plantar erythrodysesthesia (PPE) (39%), fatigue (36%), stomatitis (21%), and infusion-reaction (20%).	
GBM	GSH	GSH tagged PLGA (50:50) nanoparticles	Paclitaxel, coumarin-6	IP injection of 5 mg/kg of coumarin-6 in PLGA nanoparticles (240 nm, PDI: 0.74) decorated with 2% glutathione resulted in enhanced fluorescence of coumarin-6 in the brain and <i>in vitro</i> efficacy in RG2 glioma cells.	(Geldenhuys et al., 2011)
Malignant glioma	GSH	GSH decorated pegylated liposomes	Ribavirin	G-technology: Using microdialysis, enhanced brain uptake <i>in vivo</i> of ribavirin was demonstrated that correlated with the increasing amounts of GSH coating.	(Maussang et al., 2016)
Multiple sclerosis	GSH	GSH decorated pegylated liposomes	Methylprednisolone (MP) hemisuccinate	G-technology (Phase I study , 2B3-201): A double-blind, three-way cross over study in 18 healthy male subjects followed by a part 2 of the study that was an open-label infusion of 2B3-201 at different doses, exploring pre-treatment with antihistamines and different infusion schedules in another 18 healthy male subjects, and a cross-over study in six healthy female subjects. The most frequent recorded AE was an infusion related reaction (89%) and 2B3-201 was shown to have a plasma half-life between 24 and 37 h and caused a prolonged decrease in the lymphocyte count, adrenocorticotrophic hormone and osteocalcin, and a rise in fasting glucose.	(Kanhai et al., 2018)
AD	GLUT-1	Mannose or Mannose and Pen or Mannose and RVG decorated liposomes; DOTAP: DOPE: Chol: DSPE-PEG ₂₀₀₀ -CPP, DSPE-PEG-MAN (45: 45: 2: 4: 4 mol %)	BDNF gene	Plasmid encoding BDNF (pBDNF) was complexed with chitosan (30 kDa, N/P: 5:1) prior to encapsulation inside liposomes. Injection of MAN decorated liposomes with a dye resulted in 4.6% of ID after 12h. Pen and MAN decorated particles resulted in high brain levels up to 10% of ID/g of tissue in C57BL/6 mice. Liposomes entrapping the pBDNF-chitosan complex were given IV at 40 µg pBDNF/100 g body weight and mice were housed for 5 days before harvesting organs. Both dual modified treated mice	(Arora et al., 2020)

				exhibited 1.7 times higher BDNF levels compared to baseline levels.	
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Key; AD: Alzheimer’s disease, AE: adverse effect, BCBM: breast cancer patients with brain metastasis, BDNF: brain-derived neurotrophic factor, Chol: Cholesterol, CPP: cell penetrating peptide, DOPE: Dioleoyl-sn-glycero-3-phosphoethanolamine, DOTAP: dioleoyl-3-trimethylammonium propane chloride, GBM: glioblastoma multiforme, GLUT-1: Hexose carrier, GSH: glutathione, HGG: high grade glioma, IP: intraperitoneal, IV: intravenous, MAN: Mannose, MP: methylprednisolone hemisuccinate, N/P: Nitrogen to phosphate ratio, PDI: polydispersity, Pen: Penetratin (Cell penetrating peptide, RQIKIWFQNRRMKWKKGG) PFS: progression free survival, PLGA: poly(lactide-co-glycolide), PR: partial response, Ref.: reference, RVG: rabies virus glycoprotein (Cell penetrating peptide, YTIWMPENPRPGTPCDIFTNSRGKRASNG), SD: stable disease.

17.4.4 Receptor-Mediated Transcytosis for Brain Delivery.

Most proteins in the plasma are precluded from entry to the brain due to the hydrophilicity, size, and hydrogen bonding potential (Lalatsa et al., 2014) evident by the lower protein content of the CSF compared to plasma. The BBB expresses many receptors on its surface for the uptake of proteins such as insulin or transferrin. However, here it is important to state there is no receptor that is exclusively expressed on the surface of the brain capillary endothelial cells and that is not expressed in other parts of the body. Utilising receptor mediated transcytosis (RMT), large molecular weight compounds, macromolecules such as peptides and proteins, as well as particulate formulations (such as a drug conjugates, antibody conjugates, himeric proteins, antibodies, and functionalised nanoparticles) can be endocytosed and gain entry across the BBB.

Although the mechanism of action has not been well characterised, the process involves the binding of the ligand to its specific membrane receptor on the cell surface resulting in the modification of the receptor protein triggering the formation of invaginations that can be clathrin coated. These endocytotic vesicles may fuse with an endosome (pre-lysosomal compartment with an acidic pH) and dissociation of the ligand from the receptor takes place, allowing the free receptor to be recycled to the cell surface (Kreuter, 2014).

Table 17.3 Substrates and receptors for RMT across the BBB

Transport System (Receptor)	Abbreviations	Substrate
Insulin	Insulin	Insulin
Insulin-like growth factors	Insulin	Insulin growth factor I/II (IGF I/II)
Transferrin	TfR	Transferrin, Lactoferrin
Melanotransferrin	MTfR	Melanotransferrin
Leptin	Leptin	Leptin
Tumour Necrosis Factor	TNF α	TNF α
Epidermal Growth Factor	EGF	EGF
Immunoglobulin G	IgG	IgG
Interleukin	IL	IL1 α , IL1 β , IL6
Apolipoprotein E	ApoER2	Lipoproteins and ApoE bound molecules/particles
LDL-receptor-related protein 1 and 2	LRP1	Lipoproteins, Amyloid- β , Lactoferrin, Angiopep-2
	LRP2	ApoE, Melanotransferrin
Diphtheria toxin receptor	DTR	

There is a range of receptor proteins that can be exploited for transport as shown in Table 17.3. Transferrin receptors, which are diffusely distributed over the entire plasma membrane, migrate to coated pits only after binding to their ligand. The low-density lipoprotein (LDL) receptors are predominately localised at the membrane surface where coated pits are found even when a ligand is not bound to them (Bickel et al., 2001) and the LRP1 is able to transport compounds in a bidirectional way (Herz & Marschang, 2003) and are overexpressed on the surface of cancer cells such as malignant astrocytomas (R. Q. Huang et al., 2010). Ligand containing vesicles can be either exocytosed leading to the transport across the BBB, fused with a lysosome leading to intracellular degradation (Broadwell et al., 1988), or can bind to a second intracellular receptor as in the case of the transfer of iron from transferrin to intracellular ferritin (Willingham et al., 1984). Another intracellular pathway may involve trafficking of endosomes, containing intact receptor ligand, to the inner saccule of the Golgi complex, where the enzymes can cause dissociation of the ligand from the receptor, and the separated ligand may then be exported in vesicles destined for lysosomal degradation. Exocytosis and the avoidance of the lysosomal pathway may be a special feature of the BBB compared to the other types of cells and tissues as transcytosis of several macromolecules is a homeostatic requirement. RMT across the BBB *in vivo* has been shown for macromolecules such as insulin, transferrin, cytokines, and leptin as well as nanoparticulate carriers (Table 17.3 and 17.4).

Conjugating nanoparticles to ligands for receptors (Table 17.4) able to transport molecules across the BBB has been widely exploited recently and remains one of most successful methods to transfer therapeutics in the brain resulting in technologies in advanced stages of clinical development [e.g. successful completion of Phase II studies for ANG1005, a peptide ligand (Angiopep-2; TFFYGGSRGKRNNFKTEEY) for LRP1 conjugated to paclitaxel (Angiochem Ltd), indicated for breast cancer patients with recurrent brain metastases (BCBM patients)]. Angiopeps are a family of 19 amino acids peptides, derived from the Kunitz domain of aprotinin (Demeule et al., 2008). Angiopep-2, exhibited higher transcytosis capacity and parenchymal accumulation than do transferrin, lactoferrin, and avidin, while the transport of conjugated compounds is unaffected by efflux as the peptide is not a substrate of P-gp efflux pumps (Demeule et al., 2008). Apart from low molecular weight anticancer drug conjugates (doxorubicin (ANG1007), and etoposide (ANG1009)), Angiopep-2 conjugates with peptides such as the tridecapeptide Neurotensin (ANG2002) are under preclinical development (Demeule et al., 2014) for pain management. Angiopep-2 inhibits competitively other substrates (radioiodinated methylamine-activated α 2M) for LRP-1 in a dose-dependent manner, but does not completely displace binding even at high concentrations possibly because tested substrate binds to different binding cluster regions than other LRP1 ligands, notably the first cluster of complement-like repeats (Demeule et al., 2014). Competitive binding studies revealed that the calculated IC_{50} for ANG2002 (13.0 nM; 95% CI, 11.6–14.5) was similar to that of the endogenous NT peptide on CHO-K1 cells stably expressing human NTS1 (14.8 nM; 95% CI, 13.6–16.0) (Demeule et al., 2014). ANG2002 conserved its capacity to activate the G proteins Gq, GoA, and G13 through binding to NTS1 G-protein coupled receptors resulting in a rapid and sustained increase in ERK1/2 phosphorylation. Following an *in-situ* brain perfusion method, a time-dependent linear increase in the distribution volume (V_d) of [^{125}I]-ANG2002 which in total brain homogenate after 4 minutes of perfusion was 62.4 ± 0.7 ml/100 g brain that was significantly greater than that of the NT native peptide (7.4 ± 1.4 ml/100 g), providing *in vivo* evidence in support of ANG2002 penetration into the brain (Demeule et al., 2014). The BBB influx rate constant (K_{in}), was 10-fold greater for ANG2002 (2.7×10^{-3} ml/s/g) than for unconjugated NT (2.7×10^{-4} ml/s/g), while the [^{14}C]-inulin control in the presence of ANG2002 was unable to penetrate brain tissue (Demeule et al., 2014). In acute pain models (tail flick and hot plate assays), peak antinociception consistently occurred 15–30 minutes after injection and at the peak of response, ANG2002 caused significant increases in hot-plate latency, producing approximately 90% of the maximum possible effect

(MPE) at the highest dose. Mice receiving buprenorphine (1 mg/kg subcutaneously) also exhibited an increase in their reaction times (~55% MPE). Intravenous administration an equimolar dose of the non-brain-penetrant neurotensin intravenously was ineffective in reducing the nociceptive behaviours to noxious thermal stimulation in these models. Combining angiopep-2 with trastuzumab (anti-HER-2 monoclonal antibody (mAb), ANG4043) retained the *in vitro* binding affinity for the HER-2 receptor and potency in BT-474 breast ductal carcinoma cells (Regina et al., 2015). ANG4043 binds LRP1 clusters and after intracarotid delivery, it penetrates the BBB with a rate of entry (K_{in}) of 1.6×10^{-3} mL/g/s, while survival of mice with intracranially implanted BT-474 xenografts was increased (Regina et al., 2015).

LRP receptor is expressed in the cerebellum, on neuronal cells, and in astrocytes and is over expressed in malignant astrocytomas, especially glioblastomas (Yamamoto et al., 1997). Based on these encouraging results for conjugates of small molecular weight drugs and larger peptides and antibodies, Angiopep-2 was tested with nanoparticulate technologies. Angiopep-2 functionalised poly(ethylene glycol)-co-poly(ϵ -caprolactone) polymersomes loaded with doxorubicin compared with doxorubicin loaded polymersomes demonstrated significantly higher cellular uptake and stronger cytotoxicity in C6 cells *in vitro*, while *in vivo* pharmacokinetics and brain distribution experiments revealed that Ang-PS-DOX achieved a more extensive distribution and more abundant accumulation in glioma cells than PS-DOX (Lu et al., 2017). Moreover, the survival time of glioma-bearing rats was prolonged compared with those treated with non-functionalised doxorubicin polymersomes or a solution of free doxorubicin. The percentage of injected dose (% ID) was approximately at 0.1% in the cortex and left striatum, while 0.25% ID was recovered from intracranial tumours, while a near half %ID was available respectively for unfunctionalized polymersomes (Lu et al., 2017). Similarly, survival of glioma bearing rats was 15.8% longer with doxorubicin, 46.7% for doxorubicin polymersomes and 113% for Angiopep-2 doxorubicin loaded polymersomes (Lu et al., 2017). Some selected examples of nanoparticles are presented in Table 17.4 and ease of modification with Angiopep-2 with standard maleimide protocols allows their use with a wide range of particulate formulations. D-analogues of Angiopep-2 showed better stability *in vitro* and *in vivo* and can show better potential for brain targeting (Xie et al., 2021) (Wei et al., 2014).

Low density lipoprotein receptor (LDLR) binds lipoprotein particles carrying apolipoprotein E (ApoE) and apolipoprotein B100 (ApoB100) and internalise them by endocytosis (Spencer & Verma, 2007). Lipoprotein receptor related protein (LRP) is a multifunctional protein which can interact with a great variety of ligands including ApoE, tissue plasminogen activator, lactoferrin, amyloid precursor protein (APP) and others (Rebeck et al., 1993) mediating their endocytosis. The LRP1 receptor has also been involved in the transport of melanotransferrin across the BBB (Karkan et al., 2008). LRP1 is a higher capacity receptor compared to transferrin (TfR). The LRP1 was targeted for the transport of doxorubicin conjugates to melanotransferrin which resulted in increased survival in intracranial tumour mouse model (Karkan et al., 2008). Lactoferrin is another interesting vector for targeting the LRP1 as it is normally present at very low physiological levels and is a substrate for both the LRP1 and TfR receptors. This was exemplified by conjugating lactoferrin via a polyethylene glycol (PEG) spacer to polyamidoamine dendrimers able to complex a neurotrophic gene resulting in neuroprotective effects in a rotenone-induced rat model of Parkinson's disease (R. Huang, W. Ke, L. Han, et al., 2010).

A number of studies have exploited ligand peptide domains instead of using the whole ligand and have achieved promising results. The administration of a dipalmitoylated ApoE-derived neuropeptide conjugated to pegylated immunoliposomes resulted in uptake into brain capillary endothelial cells (Sauer et al., 2006). A lentivirus encoding for a fusion protein comprising human glucocerebrosidase and a 38 amino acid LDLR binding domain of ApoB or the 17 amino acid binding domain of ApoE

was administered intraperitoneally to mice, forming a depot in the liver secreting the fusion protein leading to glucocerebrosidase activity in the brain (Spencer & Verma, 2007)

Table 17.4 Examples of receptor-mediated transported nanomedicines

Indication	Receptor	Nanoparticulate System	Drug/Therapeutic	<i>In vitro/In vivo</i> findings	Ref.
Glioma	LRP-1	Angiopep-2 - poly(ethylene glycol)-copoly(ϵ -caprolactone) polymersomes	DOX	The percentage of injected dose (% ID) was approximately at 0.1% in the cortex and left striatum, while 0.25% ID was recovered from intracranial tumours, while a near half %ID was available respectively for unfunctionalized polymersomes	(Lu et al., 2017)
BCBM	LRP-1	Angiopep-2 PEG-PLA micelles	LPTN PAX	SKBr-3 (Her-2 positive breast cancer) orthotopic nude mouse model of BCBM showed an enhanced survival (56 days) compared to unfunctionalized micelles (47 days). A ratio of LPTN:PAX of 2:1 was optimal for drug encapsulation, loading and efficacy. Ascites and hepatomegaly were observed in the group treated with non-functionalised micelles.	(Lu et al., 2022)
Glioma	LRP-1	Angiopep-2 MSN or Angiopep-2 liposomes (DSPC:DSPE-PEG:Chol) loaded with MSN	PAX	The mean survival time of C6 glioma bearing rats treated with saline, PAX, MSN-PAX, LP-MSN-PAX, and ANG-LP-MSN-PAX treatment groups were 16.83 ± 1.32 , 18.50 ± 1.37 , 9.83 ± 1.94 , 23.66 ± 2.25 and 26.83 ± 2.22 days, respectively. No significant difference was observed between MSN-PAX and PAX treatment, indicating the low permeability of drugs across the BBB. The average tumour volume of ANG-LP-MSN-PAX, LP-MSN-PAX, MSN-PAX, and PAX were 4.52 mm^3 , 7.70 mm^3 , 19.47 mm^3 , and 23.01 mm^3 , respectively, while that of the negative control group was 57.11 mm^3 .	(Zhu et al., 2021)
GBM	LRP-1	Angiopep-2 Exosome mimetics (EM)	DTX	Angiopep-2 functionalised DTX-loaded EM (5mg/kg) showed reduced absorption of serum proteins and phagocytosis by macrophages, enhanced BBB penetration ability and targeting ability to the GBM.	(Wu, Li, Wang, et al., 2021)
GBM	LRP-1	Angiopep-2 DSPE-PEG micelles	PAX, DiR	Both D Angiopep-PEG-DSPE and L Angiopep-PEG-DSPE micelles compared to mPEG-DSPE micelles significantly increased dye distribution in the brain. No perceptible difference of dye distribution has been recognized in other organs. D Angiopep demonstrated lower uptake efficiency in bEnd.3 and U87 cells than L Angiopep, suggestive of lower binding affinity to LRP-1 of the d-peptide. D Angiopep was resistant to proteolysis in fresh rat blood serum, while more than 85% of L Angiopep disappeared within 2 h. Endocytosed D Angiopep and L Angiopep were found to be colocalized with lysosomal compartments of bEnd.3 cells, indicating that susceptibility to proteolysis of L Angiopep in the BBB may further attenuate its transcytosis efficiency.	(Wei et al., 2014)
GBM	LRP-1	Angiopep-2 SPIONS	Cy7	Both D Angiopep-2 and L Angiopep-2 modified Cy7-SPIONS probes displayed excellent tumour-homing properties and barrier penetrating abilities <i>in vitro</i> , and both could mediate precise aggregation of the nanoprobe at gliomas sites in <i>in vivo</i> magnetic resonance	(Xie et al., 2021)

				imaging (MRI) and ex vivo near-infrared (NIR) fluorescence imaging. However, compared with ¹ Angiopep-2, ² Angiopep-2 Cy7-SPIONs exhibited better enhanced MR imaging effects.	
PD	LRP-1, TfR	Lf-PEG ₃₄₀₀ -PAMAM dendrimers (generation 5)	pEGFP-N2 plasmid coding green fluorescent protein (GFP) or GDNF	Lactoferrin conjugated via a PEG spacer to polyamidoamine dendrimers delivered a neurotrophic factor gene to a rotenone-induced PD rat model with neuroprotective effects. Doubling of GDNF amounts for lactoferrin compared to unfunctionalized particles and significantly higher levels from Tf modified particles.	(R. Huang, W. Ke, L. Han, et al., 2010; R. Huang, W. Ke, Y. Liu, et al., 2010)
Pain, HGG	LDL-1 / Poloxamer 188 or Tween 80	Surfactant overcoated PLGA or PCBA nanoparticles	Loperamide, Dalargin, Doxorubicin NGF	Overcoated nanoparticles with surfactant adsorb (non-covalently) ApoE from the blood that allows permeation across LDL-1 receptor. Increase in antinociception as measured by the tail flick assay with dalargin and loperamide. Decrease in tumour blood vessel density with doxorubicin. Decrease in extrapyramidal symptoms and Parkinson's symptoms in a Parkinson's Disease mouse model	(Aliautdin et al., 1996; Alyautdin et al., 1997; Gelperina et al., 2002; Gulyaev et al., 1999; Petri et al., 2007; Schroeder et al., 1998)
Glioma	LDL-1 / Tween 80	Solid lipid nanoparticles (Compritrol® 888 ATO)	Edelfosine	Oral administration of drug-loaded SLN in NMRI nude mice bearing a C6 glioma xenograft tumour induced a significant reduction in tumour growth 14 days after the beginning of the treatment.	(Estrella-Hermoso de Mendoza et al., 2011)
PD	TfR	Anti-TfR monoclonal antibody (mAb) (OX26) via a polyethylene glycol spacer to immunoliposomes	Glial-derived neurotrophic factor (GDNF)	Apomorphine-induced contralateral rotation was reduced 87% by THL gene therapy; amphetamine-induced ipsilateral rotation was reduced 90% by THL gene therapy; whisker-induced forelimb placement abnormalities were reduced 77% with THL gene therapy. The improvement in neurobehavior correlated with a lasting 77% increase in striatal tyrosine hydroxylase enzyme activity, relative to saline treated rats. Complete recovery of neurotoxin-induced PD in rats	(Zhang et al., 2009; Zhang & Pardridge, 2009)
Glioma	TfR	Anti-TfR monoclonal antibody (mAb) (OX26) via a polyethylene glycol	shRNA plasmid (clone 952)	90% knockdown of luciferase gene expression in the brain tumour following intravenous administration of clone 952 plasmid DNA encapsulated in TfR targeted THR.	(Pardridge, 2007b)

		spacer to immunoliposomes			
PD	Insulin	HIR mAbs-PEG ₂₀₀₀ -liposomes	siRNA (tyrosine hydrolase)	Intracerebral injection of the neurotoxin, 6-hydroxy-dopamine, into the medium forebrain bundle results in chemical lesions of the nigral-striatal dopaminergic track and was used as the experimental Parkinson's disease (PD) rat model. The combined use of tissue-specific promoters and THL delivery technology allows for localization of the expression <i>in vivo</i> of the therapeutic gene to the specific organ or tissue type.	(Pardridge, 2007b)
-	Leptin	Leptin30-PEG ₃₄₀₀ -DGL	Red fluorescent protein (RFP) plasmid and pGL2-control vector	Intravenous administration results in relatively high gene transfection efficiency both <i>in vitro</i> and <i>in vivo</i> with low cytotoxicity. The luciferase expression of the DGL-PEG-Leptin30/pGL2 NPs in the brain was 1160 units/mg protein, about 1-fold higher than that of the DGL/pGL2 NPs (639 units/mg protein) and DGL-PEG/pGL2 NPs (715 units/mg protein)	(Liu et al., 2010)
PD	Leptin	Leptin-phosphatidic acid liposomes (DHDP:Chol:PA)	Resveratrol and epigallocatechin gallate (EGCG)	Leptin liposomes enhanced their ability to permeate the BBB and cellular uptake (SH-SY5Y cells). Immunofluorescence and western blot analysis also revealed that RES and EGCG encapsulated into liposomes reduce the apoptosis promoter protein Bcl-2 associated X protein and α -synuclein, and enhancement in the apoptosis inhibitor protein B cell lymphoma 2, tyrosine hydroxylase, and the dopamine transporter.	(Kuo et al., 2021)

Key; BBB: blood-brain barrier, BCBM: breast cancer to brain metastatic tumour, Chol: cholesterol, Cy5: Cyanine 5 carboxylic acid (chloride), Cy7: Indocyanine, DGL: Poly-1-lysine dendrimer, DHDP: 1,2-distearoyl-sn-glycero-3-phosphocholine, dihexadecyl phosphate, Dox: doxorubicin, DSPC: Distearoylphosphatidylcholine, DSPE-PEG: 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) 2000, DTX: docetaxel, EGCG: epigallocatechin gallate, GBM: Glioblastoma multiforme, GDNF: glial-derived neurotrophic factor, % ID: percentage of injected dose, Leptin 30: YQQVLTSLPSQNVLQIANDLENLRDLLHLLC, Lf: lactoferrin, LRP-1: LDL-receptor-related protein 1, LPTN: lapatinib, PA: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate, PAMAM: Polyamidoamine dendrimers, RES: Resveratrol, MSN: Mesoporous silica nanoparticles, PAX: paclitaxel, PD: Parkinson's disease SPIONs: superparamagnetic iron oxide nanoparticles, Tf: transferrin, Tfr: transferrin receptor, THL: Trojan-horse liposomes.

Conjugation of therapeutic compounds to specific transferrin and insulin receptor antibodies or to the relevant endogenous ligand allows their permeation across the BBB, forming conjugates that are termed as Molecular Trojan Horses (Boado & Pardridge, 2017; Chang et al., 2017; Pardridge, 2007a). The Tfr (transferrin receptor) mediates the uptake of transferrin bound iron and ubiquitously expressed throughout the body (liver, spleen, lung, and brain). Thus, the Tfr lacks selectivity/specificity for brain targeting, while competition of transferrin (Tf) conjugates with high levels of endogenous transferrin makes its use as a brain delivery vector problematic. However, combining Tfr targeting ligand with brain specific gene promoters provided sufficient specificity and delivered dose to allow for gene expression only in the brain (Shi et al., 2001). Covalently coupling an anti-Tfr monoclonal antibody (mAb) (OX26) via a polyethylene glycol spacer to immunoliposomes loaded with glial-derived neurotrophic factor results in complete recovery of neurotoxin-induced Parkinson's disease in rats (Zhang & Pardridge, 2009). OX26 antibody conjugates have been shown to localise principally within the brain endothelial cells and not in the post-capillary compartment, presumably because dissociation of this antibody from its specific receptor may be difficult due to the high affinity of the antibody for the Tfr (Moos & Morgan, 2001). Thus, antibodies with a lower binding affinity would be preferred (Moos & Morgan, 2001). Improvements in the design of anti-TFR antibodies have enabled a fused single-chain therapeutic antibody for the treatment of Alzheimer's disease with enhanced brain levels (3.5% of injected dose per g of brain) compared to antibody alone or fused with a mAb with no Tfr

specificity (0.06% of injected dose per g of brain) (Boado, Zhou, et al., 2010; Zhou et al., 2011). A bispecific low affinity anti-TfR antibody linked to a high affinity anti- β secretase (BACE1) antibody was able to reduce the production of prone to aggregation A β peptides, and thus decrease amyloid plaque formation slowing Alzheimer's disease progression resulting in a 10-fold enhanced levels (Yu et al., 2011).

The HIR (human insulin receptor) has also been used as an individual target or in combination with the TfR for brain delivery of nanoparticles even though there is a potential for such methods to interfere with insulin metabolism. HIR or TfR mediated uptake has been achieved using a variety of conjugates, notably: vasoactive intestinal peptide conjugated to TfR mAbs (VIP-TR mAb) (Bickel et al., 1993), brain derived neurotrophic factor (Zhang & Pardridge, 2001) or fibroblast growth factor-2 (Wu et al., 2002) conjugated to the HIR mAbs (BDNF-HIR mAb or FGF2-HIR mAb respectively), epidermal growth factor (EGF) (Zhang et al., 2004) or amyloid β 1-40 peptide (Kurihara & Pardridge, 2000) or siRNA (Xia et al., 2009) conjugated to the TfR mAb. In addition fusion proteins between β -galactosidase (Zhang & Pardridge, 2005) and neurotrophin (Boado & Pardridge, 2009) and the HIR mAb have been produced and patents have been filed protecting a bifunctional fusion antibody construct comprising the HIR mAb and antibodies for other BBB specific receptors (Pardridge & Boado, 2009, 2010a, 2010b, 2011).

When chimeric HIR-mAbs fused with a tumour necrosis factor decoy receptor were administered intravenously to Rhesus monkeys, 3% of the intravenous radiolabel dose (15mg kg^{-1}) was found in the brain, approximately 30% higher than that of a nonspecific fusion protein (Boado, Hui, et al., 2010). However quite high levels (45%) of the dose were detected in the liver 2 hours after dosing (Boado, Hui, et al., 2010). The HIR mAbs have also been used to deliver particulate (liposome encapsulated) agents across the BBB. HIR mAbs conjugated to PEG chains (2,000 Da) on liposomes have been shown to deliver siRNA specific for tyrosine hydrolase across the BBB (Pardridge, 2007b), leading to disease modification in a rat Parkinson's disease model. Ever more sophisticated delivery modalities are being employed to deliver proteins and other larger molecules to the brain and to this end, a novel trifunctional fusion antibody comprising HIR for brain entry, amyloid beta for disruption of amyloid plaques and the neonatal Fc receptor for exit out of the brain has also been developed (Boado et al., 2007).

Leptin is a 16 kDa protein produced in white peripheral adipocytes which binds to the leptin receptor in the choroid plexus and on the brain capillary endothelial cells, where it is taken up into the brain parenchyma (Banks et al., 1996; Barret et al., 2009). The leptin receptor can be saturated in obese patients that have elevated levels of leptin (Kd of the receptor is similar to normal serum levels). Leptin₆₁₋₉₀ provided the highest uptake in rats like the endogenous protein and has been explored for gene therapy. Leptin₆₁₋₉₀ decorated pegylated poly-lysine dendrimer nanoparticles complexed with DNA showed higher uptake in the brain compared to control nanoparticles and the attendant increased gene expression in the brain (Barrett et al., 2009).

Earlier research concentrated on receptors that transport large endogenous molecules to the brain such as transferrin and human insulin. However, current studies suggest that even a signalling receptor such as the nicotinic acetylcholine receptor may be used as a portal for brain delivery. This receptor was identified as a possible mechanism for uptake across the BBB of complexes of a 29 amino acid peptide derived from the rabies glycoprotein (RGV29) when linked conjugated via a short triglycine spacer to a D-arginine-9-mer with siRNA (Kumar et al., 2007). Uptake of these complexes was blocked by bungarotoxin, a substrate for the nicotine acetylcholine receptor, indicating that RVG29 entered the brain via this receptor and particularly involving the $\alpha 7$ subunit of this receptor (Kumar et al., 2007).

Attachment of RVG29 to cationic liposomal siRNA complexes resulted in cellular prior protein gene silencing (Pulford et al., 2010). However, as bungarotoxin is a substrate of GABA_B and the uptake of the RVG29 conjugated PAMAM dendrimers gene complexes is not able to be blocked by nicotinic agonists and antagonists, GABA_B receptor involvement in the uptake cannot be excluded (Wilkins et al., 2008).

Non-toxic mutants of diphtheria toxin employed for human vaccination have been proposed as vectors for brain delivery. Fusing the receptor-specific protein vector CRM 197 with horseradish peroxidase or functionalising liposomes entrapping horseradish peroxidase enabled the latter's transcytosis across the BBB in a guinea pig model (Schenk et al., 2012). However, it is important to note that this strategy is unsuitable for multiple administrations as potential immunogenicity problems are of concern.

A key characteristic of any successful strategy is that the vector has sufficient high affinity for the receptor, while also allowing also release of the cargo in the brain parenchyma. The physiological levels of the endogenous ligand needs to be carefully considered as the endogenous ligand should not compete with the delivery vector for receptor occupancy at the BBB. This is the reason why although transferrin was highly researched earlier has not yielded successful clinical products. Transferrin plasma concentration is more than 1,000-fold higher than the K_d value (5.6 nM). The vector conjugate should have a high receptor affinity and the type of linker or spacer used may affect the receptor affinity (as it can affect release). The brain uptake of the vector conjugate should be high enough to allow for a therapeutic dose to be administered after correction for the amount of drug contained in the brain blood capillaries. Uptake of 2% of the injected dose per gram of brain (mouse) has been suggested as a reasonable target (W. M. Pardridge, 2010). Additionally, vectors with pharmacologically activity like insulin are not ideal. Finally, ideally the receptor should be specifically expressed in the brain capillary endothelium to target the conjugate exclusively to the brain, eliminating loss of dose in the periphery and any unwanted peripheral adverse effects. However, brain receptor exclusivity is not usual.

Transcytosis involves five major stages: binding, endocytosis, trafficking, exocytosis, and unbinding. Efficient transcytosis requires the formation of ligand/receptor bonds that last enough for it to be trafficked across; yet, the higher the ligand binding energy, the lower is its ability to detach once across to the other side. Therefore, a balance is required to form and maintain not only sufficiently strong bonds to enable binding and endocytosis but also a sufficiently weak bond to allow unbinding and release. Permeability across the BBB can be enhanced by multivalency i.e. the expression of multiple ligands on the surface of the nanoparticle (Gao et al., 2013) or potentially more than substrates able to target different high capacity receptors. Raymond's group reported a high multivalent effect for the divalent constructs of TAT, TP10 and pAnt-(43-58) (cell-penetrating peptides – [see 17.4.5 Adsorptive-Mediated Transcytosis section](#)) (Eggimann et al., 2013). Statistical mechanical modelling has been described on how multivalency can be exploited to achieve selectivity i.e., binding only to targets bearing several receptors within a specified range (Liu et al., 2020). A synthetic multivalent system with tuneable avidity to LRP1 showed that high-avidity cargo biases the LRP1 towards internalisation associated with fast degradation (LRP1 enters the cells and gets trafficked to endosomes and lysosomes where it is degraded), while mid-avidity augments the formation of syndapin-2 tubular carriers promoting fast shuttling of polymersomes across the BBB (Tian et al., 2020). These pathways are driven by cargo avidity, and thus this needs to be carefully tuned. Intermediate ligand numbers push more to the syndapin-2 pathway associated with tubular deformations and thus higher uptake, while the higher number of ligands and avidity pushes the cargo more toward endosomal sorting (Tian et al., 2020).

17.4.5 Adsorptive-Mediated Transcytosis for Brain Delivery.

While receptor-mediated transcytosis involves specific plasma membrane receptors, cationic large molecular weight biopharmaceuticals can be taken up by the brain via adsorptive mediated transcytosis (AMT). AMT requires an excess positive charge on the molecule or particle at physiological pH, which allows it to interact electrostatically with anionic sites on the cell surface, i.e. acidic glycoproteins (type IV collagen, laminin, fibronectin and heparin sulphate) (Vorbrodt, 1989), triggering endocytosis and subsequent transcytosis (Sauer et al., 2005). The processes following endocytosis are similar to the processes associated with RMT. However, AMT has a higher capacity for transport compared to RMT. Cationised albumin is known to utilise this pathway to gain entry to the brain (Kumagai et al., 1987; Pardridge, Triguero, et al., 1990), along with avidin (Pardridge & Boado, 1991), histone (Pardridge et al., 1989), cationised polyclonal bovine immunoglobulin (Triguero et al., 1989), E-2078 (small dynorphin-like basic peptide) (Terasaki et al., 1991) and the cell penetrating peptides HIV transactivator of transcription (TAT) protein (Frankel & Pabo, 1988) and other arginine-rich peptides such as SynB5 (RGGRLAYLRRRWAVLGR) and pAnt-(43-58) (RQIKIWFQNRRMKWKK) (Drin et al., 2003). The arginine content of these oligomers is a critical factor (Schmidt et al.) and the important structural features of guanidinium-rich cell-penetrating vectors are now better understood (Wender et al., 2008). However, the concentration of cationic peptides in the brain may be limited by the fact that cationic agents are more readily taken up by the liver and kidney, so that the actual mass taken into the brain is minimal - less than 0.1% of intravenously injected dose (Lee & Pardridge, 2001). To prevent peripheral organ uptake, workers have tried masking the cell-penetrating vector with another oligopeptide, which is designed to be cleaved off at the target tissue by specific extracellular proteases, and expose the cationic vector to promote absorption (Jiang et al., 2004). Lipidation of the cationic polypeptide has also been used as a strategy to enhance transcytosis of a myristoylated polyarginine vector (Pham et al., 2005). Neurotrophic factors covalently linked to naturally occurring polyamines such as putrescine, spermidine and spermine have been also shown to have increased BBB permeability (Poduslo et al., 1998). Examples of nanoparticles for adsorptive-mediated transcytosis are described in Table 17.5. Combining potentially cell-penetrating peptides for receptor and adsorptive mediated transcytosis can also be exploited for enhancing BBB permeability of nanoparticles, however, toxicity of this particles needs to be carefully controlled.

Table 17.5 Examples of adsorptive-mediated transported nanomedicines.

Indication	CPP	Nanoparticulate System	Drug/Therapeutic	<i>In vitro/In vivo</i> findings	Ref.
BCBM	ACUPA and cyclic TT1	Poly(lactic-co-glycolic acid)-poly(ϵ -carbobenzoxymethyl-L-lysine) nanoparticles	DOX LPTN	Integrin-targeting RGD-modified NPs in BTB endothelial cells and displayed about 4.57-fold stronger penetration through the BCBM-associated BTB as compared to the normal BBB. Synergistic antitumour effect of DOX and LPTN.	(Lu et al., 2017)
Brain tumours	TAT	MPEG ₂₀₀₀ -PCL-TAT micelles	siRNA	Intranasal and intravenous administration resulted in ~2 and ~0.2 % ID of Alexa labelled dextran (10 kDa) in the brain. Only nose-to-brain delivery indicated gene expression <i>in vivo</i> .	(Kanzawa et al., 2013)
Alzheimer's disease	K16ApoE	PLGA	Curcumin	The K16ApoE-Targeted nanoparticles demonstrated specific targeting of vasculotropic DutchA β 40 peptide accumulated in the cerebral vasculature. Moreover, K16ApoE-Targeted nanoparticles demonstrated significantly greater uptake into brain and provided specific MRI contrast to detect brain amyloid plaques.	(Ahlshede et al., 2019)

Alzheimer's disease	TAT	PLGA	Insulin	Nanoparticles deliver insulin into brain via the nasal route with a total brain delivery efficiency of 6%.	(Yan et al., 2013)
Glioma	Cationic albumin	Albumin (HSA) plus cationic- (c-HSA) or mannose-modified-albumin (m-HSA)	Doxorubicin	Cationic and mannose modified HSA particles exhibited the most prominent performances in transport across the bEnd.3 cell monolayer and uptake in bEnd.3 cells as well as U87MG glioblastoma cells and spheroids. Also, these particles were localized to a greater extent in brain glioma compared to naïve HSA NPs. Orthotopic glioma-bearing mice treated with cationic and mannose modified HSA particles displayed significantly smaller tumours than those in saline, doxorubicin or HSA particle treated mice. Adsorptive mediated transcytosis and uptake via the GLUT-1 carrier are proposed mechanisms for these dual targeted particles.	(Byeon et al., 2016)

Key; BBB: blood-brain barrier, BCBM: breast cancer to brain metastatic tumour, CPP: cell-penetrating peptide, HSA: albumin, % ID: percentage of injected dose, K16ApoE: 6 lysine residues and amino acids 151–170 of the low-density lipoprotein receptor (LDLR)-binding segment of the apolipoprotein E (ApoE) peptide, PCL: polycaprolactone, PLGA: poly(lactic-co-glycolic acid), TAT: GRKKRRQRRRP,

17.5 Exosomes for brain delivery.

Exosomes are a subset of small extracellular vesicles (EVs) that are produced by all normal and malignant cells and are present in all body fluids and are typically heterogeneous, comprising vesicles with various sizes that originate from many different cell types (Banks et al., 2020). Exosomes between 30–150 nm in diameter are of special interest because of their origin, their ability to freely circulate and infiltrate various tissues and their molecular content mirroring that of parental cells typically originating from the endosomal compartment of parent cells, where they are formed by inverse membrane invagination as intraluminal vesicles inside multivesicular bodies (MVBs) (Banks et al., 2020). When MVBs fuse with the cell membrane, exosomes are released into the extracellular space. The orientation of surface proteins in exosome membranes resembles that in the cell membrane of parent cells (Zhang et al., 2018). Exosomes serve as an intercellular communication system, shuttling messages between cells and also conveying peptides, proteins, and genetic materials from parental to recipient cells. EVs derived from erythrocytes can cross the blood–brain barrier (BBB), contain large amounts of alpha-synuclein, and may contribute to Parkinson's pathology (Matsumoto et al., 2017). As exosomes circulate freely, and appear to cross various organ barriers, attempts have been made to engineer them synthetically to deliver drugs to target tissues, including the brain. Adsorptive-Mediated Transcytosis, active efflux transport, carrier-mediated transport, and receptor-mediated transport (Transferrin Receptors, Folate Receptors, Lipoprotein receptor-related Protein, Scavenging Receptors, Interleukin-13 Receptor α 2, Insulin Receptors, Glutamate Receptors) have been reported for their uptake across the BBB (Heidarzadeh et al., 2021).

Exosome-mimetic nanovesicles have been shown to be able to be loaded with doxorubicin and exhibit suppression effects similar to exosome loaded with doxorubicin on the progress of glioblastoma (GBM) in zebrafish and *in vivo* subcutaneous and orthotopic xenografts mice models, with minimal systemic toxicity (Wu, Li, Hu, et al., 2021). Exosome-mimetic cell membrane nanovesicles (CMNVs), produced by extrusion with isolated C6 cell membrane fragments and decorated with cholesterol-linked T7 peptides were able to deliver 2'-O-methyl and cholesterol-TEG modified anti-microRNA-21 oligonucleotides (AMO21c) in an orthotopic mouse model of glioblastoma (Lee et al., 2022). Decorated CMNVs down-regulated miRNA-21 (miR-21) levels in glioblastoma tissue most efficiently resulting in the up-regulation of PDCD4 and PTEN, while the brain tumour size was reduced more efficiently

than in the other control groups (Lee et al., 2022). This is an emerging area of nanomedicine brain delivery and can find significant applications in cancer and inflammatory diseases.

17.5 Particle characteristics for successful brain and brain tumour delivery; pharmacokinetic and pharmacodynamic considerations.

Irrespectively of the application and potentially targeted pathway for transport across the blood-brain barrier, there are certain particle characteristics pharmacokinetic and pharmacodynamic parameters that need to be taken carefully into consideration for an effective and translatable nanoparticulate strategy (Table 17.6). Nanoparticulate delivery systems should be tailored in terms of their size, hydrophobicity and surface charge to avoid rapid clearance from the body and a long circulation half-life, facilitate brain permeability and also tumour or tissue targeting as required. Nanoparticles (NPs) with sizes between 15 -100 nm possess a long circulation time compared to smaller particles (10-20 nm) that are rapidly filtered via the kidneys or larger (>150nm) that are uptaken by the reticuloendothelial system (RES) (Jiang et al., 2008; Nance et al., 2012; Win & Feng, 2005). Nanoparticles of optimal size will eventually be uptaken by the liver, but they will enjoy a long circulation half-life (2-40 h) which is critical for the accumulation of the nanoparticulate system or the therapeutic across the BBB (Lalatsa, Leite, et al., 2015; Owens & Peppas, 2006).

The shape of the particles plays a critical role as long axial particles with a diameter of ~20 nm and length larger than 18 μm could remain in circulation for longer than 5 days (long enough to mechanically hinder uptake into macrophages) (Geng et al., 2007). Long axial particles as peptide nanofibers (Lalatsa, Garrett, et al., 2012; Lalatsa, Lee, et al., 2012; Lalatsa, Leite, et al., 2015; Lalatsa, Schatzlein, et al., 2015; Leite et al., 2015) and carbon nanotubes (Kafa et al., 2015) have shown promise in delivery of therapies such as peptides across an intact BBB enabling 0.4% of the intravenous injected dose to reach the brain. Between the two technologies, peptide nanofibers offer the advantage of low toxicity as are able to be completely enzymatically metabolised to degradation products naturally present within the body and the brain parenchyma as well as high specificity due to their peptide nature, and preclinical proof of concept in a murine model (Lalatsa, Schatzlein, et al., 2015).

A hydrophobic particle surface is deleterious to a long circulation half-life and coating of particles with low molecular weight surfactants such as polysorbate 80 (Wang et al., 2009) or hydrophilic polymers notable polyethylene glycol (5 kDa), chitosan or albumin can overcome this problem (Lu et al., 2005; Serrano Lopez & Lalatsa, 2013). Positively charged surfaces promote BBB permeation by physical adsorption to the endothelium with cationic particles readily taken up into the cells at the periphery of tumour spheres compared to anionic (Kim et al., 2010). Thus, the potential to maintain a high plasma concentration and interact favourably with the blood-tumour interface make nanoparticles highly useful for glioma targeting.

The drug loading efficiency is critical as nanocarriers with low payload will not result in efficient accumulation of high amounts of the payload to the brain. The drug loading efficiency is dependent not only on the size but also on the nanocarrier constituents (e.g. charge) and the specific interaction with the drug. The method of drug loading can also greatly influence the efficiency and the scale-up of the proposed system. Drugs can be encapsulated into liposomes for example via passive loading (phospholipids dispersed in an aqueous solvent containing the drug spontaneously forming bilayers separated by narrow aqueous compartments with low encapsulation efficiency. On the other hand, remote loading, achieved via drugs loaded into preformed liposomes using a transmembrane pH or salt gradient can result to high encapsulation efficiencies (80-100%) (Zucker et al., 2009). The higher encapsulation efficiency associated with remote loading increases the drug to lipid ratio for liposomes, which in turn increases the change to deliver enough drug without reaching the dose limits of the carrier inducing toxicity (Zucker et al., 2009).

Table 17.6 Nanomedicine characteristics and impact for brain tumour localisation.

Size (nm)	Small				Large
	<10 nm	< 20 nm	< 70 nm	< 100 nm	>150 nm
	Rapid glomerular filtration	Exit tumour cells more easily once internalised (reduced EPR effect)	Improved convective flow through tumour and normal brain	Permits tumour entry via EPR effect	Difficult cell entry via endocytosis – clearance by RES
Hydrophobicity	Hydrophilic	Amphiphilic	Hydrophobic		
	Increased circulation half-life	Increased BBB permeation	Rapid clearance by RES		
Surface Charge	Cationic	Uncharged	Anionic		
	Adsorptive-mediated BBB transcytosis, cell membrane disruption at high charge	Reduced charge may facilitate spread through tumour ECM	Reduced brain tumour cellular uptake in vivo		

Key: ECM: extracellular matrix, EPR: enhanced permeation and retention effect, RES: reticuloendothelial system.

When investigating nanocarriers for brain drug delivery, brain distribution is the most used method in which the total drug concentrations in plasma and brain homogenates are measured. However, as it is not possible to take multiple brain samples compared to blood samples, requires a large sacrifice of animals, while there is risk of overestimation due to the residual blood in the brain homogenates even after perfusion as some drugs might still be attached to endothelial cells in released and encapsulated forms. This measures total concentration, so it is difficult to separate encapsulated, released, unbound drug, and released plasma protein bound drug. In vivo brain microdialysis is used to characterise the influx and efflux of nanomedicines across the BBB under both physiological and pathophysiological conditions. A probe with a semipermeable membrane allows small and water-soluble solutes to cross by passive diffusion. Thus, this method only allows for continuous measurement of the released, unbound drug concentrations in plasma, brain and brain interstitial fluid over time which is pharmacologically relevant. When combined with regular blood sampling, both the rate and extent of drug release in vivo and transport at the BBB can be quantitatively assessed. However, this method is unsuitable for lipophilic compounds to extensive partition into the tubing and probe material. Tissue damage after surgical insertion of probe is possible and timing of experiments does not allow for recovery to reach reasonable levels. Cerebrospinal fluid (CSF) is also used to determine brain uptake especially in primates or humans as a direct measurement of ISF and total brain concentrations. However, CSF concentrations do not always reflect unbound drug concentration in ISF and blood contamination of CSF remains a problem. Imaging techniques such as positron emission tomography (PET), magnetic resonance imaging (MRI), computed X-ray tomography (CT) and single-photon emission computed tomography (SPECT) are increasingly used for in vivo imaging of brain tissue. These techniques usually require the use of radiotracer or fluorescently label that can adulterate the distribution of the nanomedicines. Raman spectroscopy is increasingly being researched to provide new label-free alternatives for nanomedicine imaging (Lalatsa, Garrett, et al., 2012).

17.6 Regulatory considerations

The current manufacturing methods for the majority of licensed nanomedicines remain discontinuous and face a number of problems such as high batch-to-batch variability affecting the critical quality attributes (CQAs) of the product, laborious multistep processes, need for an expert workforce, and not being easily amenable to industrial scale-up involving typically a complex process control (Osouli-Bostanabad et al., 2022). Main critical manufacturing parameters (pressure, shear force, pH, temperature etc.) need to be identified early on to ensure a strictly controlled manufacturing process. Reproducibility should also be demonstrated from multiple batches at different production scales and

ideally method of manufacture should be continuous. Microfluidics is the technology of fluid manipulation in channels with dimensions of tens of micrometres, and small volumes of liquid reagents are rapidly mixed in a microchannel in a highly controlled manner to form nanoparticles with tuneable and reproducible structure that can be tailored for drug delivery, resulting in a continuous and industrial amenable manufacturing process. Several techniques have emerged in recent years for nanomedicine manufacture; however, a paradigm shift occurred, when microfluidic strategies were employed for continuous manufacture. Largely irrespective of the nature of the process, continuous flow conditions offer clear advantages over traditional batch processes, as quantity of the product scales directly with time but does not require different reactors (easy scalability), while fixed geometries allow for a precise control of mixing conditions (reproducibility) and enable lower size dispersity, as well as, in some cases, better drug loading; moreover, fine-tuning of particle properties such as a size is possible via control of the process parameters such as flow (Osouli-Bostanabad et al., 2022).

Translating preclinical successful nanomedicines into the clinic requires careful consideration of the key quality attributes and the chemistry, manufacturing process and analytical techniques need to be of adequate quality for manufacture (Table 17.7). A clear audit trail for quality excipients and ability to scale-up remain key challenges along with industrial amenable processes.

Table 17.7 Chemistry, Manufacturing and Controls criteria for nanomedicine quality and product safety

Main Criteria	Details
Product quality	Characterisation of physicochemical properties of the end product, such as Size, charge, morphology Encapsulation efficiency and drug loading Phase transition temperature In vitro release of drug substance Leakage rate/Burst release throughout shelf-life
Control of each excipient	Full description and characterisation Manufacturing specifications Stability data
Manufacturing process and process controls	Design the manufacturing process reproducibility, purity, and sterility need to be demonstrated – also during the upscaling of the production process
Control of drug product	Assays for encapsulated and free drug substance * Assays for nanocarrier components/excipients * *Both should include degradation products
Stability	Shelf-life (physical and chemical stability as nanoparticles are prone to aggregation and drug leakage)

Currently the FDA and EMA only have a draft guideline for nanomedicines and a guideline for liposomal products (FDA, 2022). The criteria in these guidelines need to be reviewed on a case-by-case basis for other types of carriers. So preclinical nanomedicines that show promise for further development require to have proof of safe receptor biology in humans, a safe and human applicable ligand, receptor-specific binding for an acute or chronic condition, favourable pharmacokinetic profile, no modification of the active ingredient (otherwise further toxicology is required), ideally be a platform technology able to carry a wide range of therapeutics, low cost and industrially amenable scalable process, activity in animal models (although the FDA recently is no longer requiring this) and strong intellectual property protection.

17.7 Conclusions

Since the approval of Caelyx® in 1995 and the recent development of COVID-19 vaccines, much progress has been realised towards the clinical development of nanomedicines. However, the clinical translation of brain targeted nanomedicines is lagging, because of the added challenges associated with delivery to the brain and other inherent difficulties in CNS drug development. The accelerated development of nanodelivery systems for RNA therapeutics and advances in microfluidics is likely to lead to new treatments for CNS diseases. Even though a magic bullet for CNS delivery has not been realised a century later from its initial proposal, substrates are available for tuning nanoparticulate transport across the BBB. Combining safe targeting ligands with well-known and safe nanocarriers, brain-targeted nanomedicines may be capable of enhancing brain drug delivery and impacting the clinical treatment of devastating CNS diseases.

17.8 Question Box

17.8.1 Question 1

Question 1: Is multivalency beneficial for brain targeting of nanomedicines?

Answer 1: Brain endothelial capillaries control the transport of small molecules, such as glucose and amino acids, by expressing specialized solute carrier transporters on both apical (blood) and basal (brain) membranes that shuttle molecules across. Brain endothelial capillaries express a range of receptors (Table 17.3) such as transferrin, insulin, and the low-density lipoprotein receptor-related protein 1 (LRP1) as well as a range of carriers (Table 17.1) that allow transcytosis of molecules and particles. Permeability across the BBB can be enhanced by multivalency i.e., the expression of multiple ligands on the surface of the nanoparticle (Gao et al., 2013) or potentially more than substrates able to target different high-capacity receptors. So for a successful functionalised strategy, it is important to target high capacity transport systems such as the LRP1 that is expressed in neurons, astrocytes, cancer cells, and brain endothelial capillary cells and is able to bind more than 40 ligands undergoing rapid endocytosis with a half-life of less than 30 seconds (Tian et al., 2020) or adsorptive-mediated transcytosis. Utilising dual targeting has been demonstrated to near double the nanoparticle permeation as long as both carrier or receptor mediated systems are high capacity (R. Huang, W. Ke, L. Han, et al., 2010). The avidity of the functionalised particles need to be carefully tuned as the particles are multivalent and thus possess more than one binding sites. The measure of the total binding strength of a nanoparticle at every binding site is termed avidity (of functional affinity) and is determined by three factors: the binding affinity (strength of binding at a singular binding site), the valency (total number of binding sites involved) and the structural arrangement (how accessible the binding site is e.g. on the surface of PEG brush on the surface of pegylated particles). Studies using multivalent particles with tuneable avidity to LRP1 showed that high-avidity cargo biases the LRP1 towards internalisation of the receptor and endosomal trafficking and lysosomal degradation, while mid-avidity augments the formation of syndapin-2 tubular carriers that promote fast shuttling of nanoparticles across the BBB (Tian et al., 2020). These pathways are driven by cargo avidity, and thus this needs to be carefully tuned. However, additionally, for clinical translation, the complexity of functionalisation, scalability, requirement for appropriate assays able to characterise the extent (multivalency) and stability of functionalised nanoparticles and the added cost need to be carefully taken into consideration compared to the overall efficacy and targeting achieved by proposed nanomedicines.

17.8.2 Question 2

Question 2: Briefly describe a clinically successful strategy that has been used to enhance the brain targeting of a polymeric or lipidic nanomedicine.

Answer 2: G-technology (to-BBB technologies) utilises glutathione-pegylated liposomes for the delivery of doxorubicin for the treatment of brain tumours. This is carrier mediated approach utilising the glutathione carrier system expressed in brain capillary endothelial cells. Pegylated (2,000 Da) liposomes were loaded using the ammonium sulphate method to ensure high loading of doxorubicin and demonstrated similar intravenous pharmacokinetics to that of Caelyx[®] and demonstrated linear pharmacokinetic after intravenous administration up to a dose of 7mg/kg. Uptake of 2B3-101 by human brain capillary endothelial cells *in vitro* was time-, concentration- and temperature-dependent, while pegylated liposomal doxorubicin mainly remained bound to the cells (Gaillard, Appeldoorn, et al., 2014). *In vivo*, 2B3-101 and pegylated liposomal doxorubicin had a comparable plasma exposure in mice, yet brain retention 4 days after administration was higher for 2B3-101 (Gaillard, Appeldoorn, et al., 2014). 2B3-101 was overall well tolerated by athymic FVB mice with experimental human glioblastoma (luciferase transfected U87MG). In 2 independent experiments a strong inhibition of brain tumour growth was observed for 2B3-101 as measured by bioluminescence intensity. The effect of weekly administration of 5 mg/kg 2B3-101 was more pronounced compared to pegylated liposomal doxorubicin and saline. Two out of 9 animals receiving 2B3-101 showed a complete tumour regression. Twice-weekly injections of 5 mg/kg 2B3-101 again had a significant effect in inhibiting brain tumour growth compared to pegylated liposomal doxorubicin and saline, and a complete regression was observed in 1 animal treated with 2B3-101. In addition, twice-weekly dosing of 2B3-101 significantly increased the median survival time by 38.5% and 16.1% compared to saline and pegylated liposomal doxorubicin, respectively.

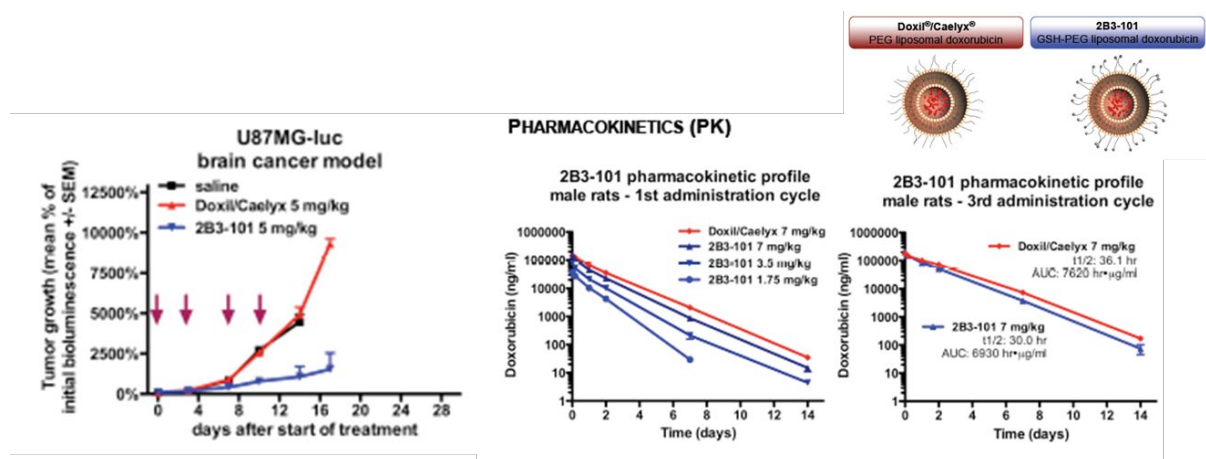


Figure 17.3 Tumour growth and pharmacokinetic profile after intravenous administration of 2B3-101. Reproduced from (Gaillard, Appeldoorn, et al., 2014)

In a phase 1/2 study, 2B3-101 was tolerated up to a dose-intensity of 15 mg/m²/week with no neuro- or cardiotoxicity. In 25 evaluable human high grade glioma (HGG) patients, 52% had stable disease (SD) as best response and 3-months progression free survival (PFS) rate of 40%. In 23 evaluable BCBM patients, 9% had partial response (PR) and 48% SD as overall best response with 3-months PFS rate of 48%. Adverse effects observed that were more significant than grade 2 involved neutropenia (41%), palmar-plantar erythrodysesthesia (PPE) (39%), fatigue (36%), stomatitis (21%), and infusion-reaction (20%). Overall, these data demonstrate that glutathione pegylated liposomal doxorubicin enhances the effective delivery of doxorubicin to brain tumours and could become a promising new therapeutic option for the treatment of brain malignancies.

17.9 References

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