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Overcoming the Blood–Brain Barrier: The Role of Nanomaterials in Treating Neurological Diseases

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Abbreviations

α 2M, alpha-2-macroglobulin; A β , amyloid β ; ABC, ATP-binding cassette; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; ALCAM, activated leukocyte cell adhesion molecule; ALS, amyotrophic lateral sclerosis; AmB, amphotericin B; AMT, adsorptive-mediated transcytosis; ANEP, anti-neuroexcitation peptide; Antp, Antennapedia; APC, antigen-presenting cell; Apo, apolipoprotein; APP, amyloid β precursor protein; ATP, adenosine triphosphate; AuNP, gold nanoparticle; BACE1, β -secretase 1; BBB, blood-brain barrier; BCRP, breast cancer related protein; BCSC, brain cancer stem cell; BCSFB, blood-cerebrospinal fluid barrier; BDNF, brain-derived neurotrophic factor; BLB, blood-labyrinthine barrier; BMEC, brain microvessel endothelial cell; BSA, bovine serum albumin; BsAb, bispecific antibody; CASK, Ca²⁺-dependent serine protein kinase; CAT, cationic amino acid transporter; CBF, cerebral blood flow; CBSA, cationic bovine serum albumin; CED, convection-enhanced delivery; CMT, carrier-mediated transport; CNS, central nervous system; CP, choroid plexus; CPP, cell penetrating peptide; CR, complement receptor; CRM, cross-reacting material; CSF, cerebrospinal fluid; CTX, chlorotoxin; CYP450, cytochrome P450; DAM, disease-associated microglia; DARPin, designed ankyrin repeat protein; DMMA, 2,3-dimethylmaleic anhydride; DON, 6-diazo-5-oxo-l-norleucine; DOPE, dioleoyl-phosphatidylethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane; DT_R, diphtheria toxin receptor; EAAT, excitatory amino acid transporter; EAE, experimental autoimmune encephalomyelitis; EBV, Epstein-Barr virus; ECE, endothelin-converting enzyme; ECM, extracellular matrix; EGF, epidermal growth factor; EMF, electromagnetic field; EO, ethylene oxide; EPR, enhanced permeability and retention; ET-1, endothelin-1; FBP, fusion sequence-based peptide; FcR, Fc receptor; FcRn, neonatal Fc receptor; FDA, U.S. Food and Drug Administration; FGF, fibroblast growth factor; FND, fluorescent nanodiamond; FR, folate receptor; FUS, focused ultrasound; GBM, glioblastoma multiforme; GLUT, glucose transporter; HIR, human insulin receptor; HIV, human immunodeficiency virus; HPMA, *N*-(2-hydroxypropyl) methacrylamide; HSA, human serum albumin; HSP, heat shock protein; ICAM, intercellular adhesion molecule; ICV, intracerebroventricular; IDE, insulin-degrading enzyme; IFN, interferon; IFP, interstitial fluid pressure; IgG, immunoglobulin G; IL, interleukin; IR, insulin receptor; iRGD, internalizing Arginine-Glycine-Aspartate; ISF, interstitial fluid; JAM, junctional adhesion molecule; LAT, large neutral amino acid transporter; LbL, layer-by-layer; LDLR, low density lipoprotein receptor; Lf, lactoferrin; LIF, leukemia inhibitory factor; LINGO-1, leucine-rich repeat and Ig-containing Nogo receptor interacting protein-1; LRP, low density lipoprotein receptor-related protein; LPS, lipopolysaccharide;

LSPR, localized surface plasmon resonance; LUV, large unilamellar vesicle; mAb, monoclonal antibody; MAP, model amphipathic peptide; MCT, monocarboxylate transporter; MDA, malondialdehyde; MDR, multidrug resistance; mGluR1, metabotropic glutamate receptor 1; MHC, major histocompatibility complex; MLV, multilamellar vesicle; MMP, matrix metalloproteinase; MNP, magnetic nanoparticle; MPS, mononuclear phagocyte system; MR, mannose receptor; MRI, magnetic resonance imaging; MRP, multidrug resistance-associated protein; MS, multiple sclerosis; MSC, myeloid suppressor cell; MTF, melanotransferrin; NEP, neprilysin; NO, nitric oxide; NOS, nitric oxide synthase; NP, nanoparticle; NVU, neurovascular unit; OSN, olfactory sensory neuron; PACA, poly(alkyl cyanoacrylate); PACAP, pituitary adenylate cyclase-activating peptide; PAH, polyallylamine hydrochloride; PAI, plasminogen activator inhibitor; PAMAM, poly(amidoamine); PBCA, poly(butyl cyanoacrylate); PCL, poly(caprolactone); PD, Parkinson's disease; PDGF, platelet-derived growth factor; PECAM, platelet-endothelial cell adhesion molecule; PEG, poly(ethylene glycol); PEI, poly(ethylenimine); PGA, poly(glutamic) acid; P-gp, P-glycoprotein; PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PO, propylene oxide; POM, pivaloyl-oxyl-methyl; PPMS, primary progressive multiple sclerosis; PS 80, polysorbate 80; PSA, prostate-specific antigen; PSS, polystyrenesulfonate; PVA, poly(vinyl alcohol); QD, quantum dot; RAGE, receptor for advanced glycation end products; RAP, receptor-associated protein; REM, rapid eye movement; RES, reticuloendothelial system; RGD, Arginine-Glycine-Aspartate; RMT, receptor-mediated transcytosis; ROS, reactive oxygen species; RRMS, relapse-remitting multiple sclerosis; RWM, round window membrane; SAS, subarachnoid space; sdAb, single domain antibody; SLN, solid lipid nanoparticle; SOD, superoxide dismutase; SPIO, superparamagnetic iron oxide; SPMS, secondary progressive multiple sclerosis; SUV, small unilamellar vesicle; Syn-B, protegrin-derived pegelin protein; TAM, tamoxifen; TAT, transactivator of transcription; TCA, tricarboxylic acid; TEER, transendothelial electrical resistance; Tf, transferrin; TfR, transferrin receptor; TGF, transforming growth factor; TM, thrombomodulin; TMC, trimethyl chitosan; TMEM30A, transmembrane protein 30A; TNF, tumor necrosis factor; tPa, tissue plasminogen activator; UCL, upconversion luminescence; UCNP, upconversion nanoparticle; USPIO, ultrasmall superparamagnetic iron oxide; VaD, vascular dementia; VCAM, vascular cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide; VSMC, vascular smooth muscle cell; WNV, West Nile Virus; ZO, zonula occludens.

1. Introduction

In the late 1800s, Paul Ehrlich, having discovered a new *in vivo* staining technology, intravenously injected colored dyes into laboratory animals and observed that all organs became stained except the brain. Meanwhile, direct injection of the same colored dyes into the brain yielded a successful staining.^[1] This seminal work led to the discovery of the blood–brain barrier (BBB), and in the over 130 years that have elapsed since then researchers have vigorously set about to uncover the fundamental biological mechanisms that underpin the BBB. The BBB is arguably the most tightly regulated of the three interfaces that separate the vascular system from the central nervous system (CNS).^[2] And for good reason. The human brain, although it comprises only ~2% of total body mass, receives up to 20% of cardiac output, and is responsible for 20–25% of the body’s oxygen and glucose consumption.^[3] This is facilitated by 100 billion capillaries, that have a combined length of 650 km and a total surface area of 20 m².^[2, 4] Cell-to-cell communication within the brain is achieved by the transmission of chemical signals (neurotransmitters and modulators) and electrical signals (synaptic potentials and action potentials) from neuron to neuron. Fundamentally, such communication involves the precise active movement of ions across CNS membranes, generated on top of passive ionic fluxes that maintain stable resting potentials.^[5] Add to this the fact that individual neurons are rarely more than 8–20 μm from a brain capillary,^[6] and it becomes evident that the BBB plays an essential role in regulating the homeostatic microenvironment of the brain.

Just as BBB integrity is crucial for the correct functioning of the CNS, it is similarly evident that BBB disruption is a key element in the progression of many brain-linked diseases. It is even hypothesized that BBB dysfunction may substantially contribute to the etiology of conditions such as Alzheimer’s disease (AD), Parkinson’s disease (PD),

amyotrophic lateral sclerosis (ALS),^[3] multiple sclerosis (MS),^[7] and several others. Although the complete pathological underpinnings of these diseases have yet to be fully elucidated, researchers are gradually beginning to derive better insights about the different ways these diseases can be treated. However, most preclinical and clinical studies to date reveal the relative lack of success investigators in the field have encountered. It has been proposed that the delivery of therapeutic drugs through the BBB is an optimal and minimally invasive strategy by which to target the brain and thus combat neurodegenerative disease.^[7] However, approximately 98% of small molecule drugs and virtually all large molecule drugs are routinely excluded from the brain.^[8] Of the therapeutics that have been successful in crossing the BBB, the greatest challenges facing clinical application include systemic cytotoxicity due to poor drug selectivity, increased BBB disruption due to the drug's pathway of entry into the brain,^[9] and insignificant brain penetration (1–4% for most CNS drugs)^[10] due to low BBB permeability and/or rapid elimination. Alternative methods exist to bypass the BBB altogether but these have exhibited problems of their own, including slow rates of drug distribution, clinical incidence of hemorrhage and CNS infection (in the case of invasive neurosurgical methods), rapid elimination of drugs by active transport, and extremely low penetration of drugs into the brain parenchyma (the parenchyma refers to the brain's functional tissue, including neurons and glial cells).^[7] Since neurological disorders contribute to approximately 12% of total deaths globally,^[11] as well as a significant proportion of morbidities and comorbidities, the need for safe and viable technologies that deliver drugs specifically to disease target regions remains an important challenge (**Figure 1**).

The main objective of this Review is to provide insight into the opportunities and challenges associated with successful therapeutic delivery to the CNS, in a format that is easily accessible to the diverse range of researchers—both new and established—active in the field of neurological disease (e.g., neuroscientists, clinicians, material scientists, chemists, and

engineers). Following an overview of BBB anatomy, physiology and pathology, we investigate various therapeutic strategies that involve either bypassing or crossing the BBB to access the CNS. We subsequently explore strategies for achieving targeted drug delivery to cells and tissues *inside* the CNS, as well as the effect of clearance mechanisms and pathways. Ultimately, our focus is on the current state-of-the-art of biological understanding and medical procedures, and the way in which nanomaterial-based approaches are advancing and facilitating these. This Review complements noteworthy existing literature that explores novel approaches to treating neurological diseases, including nanoparticle-mediated drug delivery.^[9-11, 13-16] One of the challenges in the field of bio-nano science, especially within the context of its biomedical applications (including the design of nanomaterial-based CNS therapeutics), is that the use of bio-nano standards and standardization in research remains uncommon. This can make the comparison of studies challenging, and definitive conclusions and recommendations difficult to establish.^[17, 18] Nevertheless, this Review seeks to summarize relevant design parameters wherever possible, to highlight promising strategies for achieving controlled drug delivery to the CNS.

2. Overview of BBB Anatomy and Physiology

In order to develop effective CNS drug delivery strategies, an understanding of the underlying biology is a prerequisite. This section will explore the fundamental physiological aspects of the BBB, including the different cell types that regulate BBB function (collectively called the neurovascular unit (NVU)), as well as the various barriers that play key roles in maintaining the BBB's integrity and exclusivity. In subsequent sections, CNS drug delivery strategies will be discussed in light of their relevant physiological mechanisms and interactions. For a more in-depth exploration of the BBB's physiology, there are a number of excellent reviews available.^[2, 5, 19-22]

2.1. Structure of the Neurovascular Unit (NVU)

Owing to its paramount importance in the everyday functioning of the body, the brain is the ultimate sanctuary of the CNS. Whilst the barriers between blood and parenchyma elsewhere in the body are less tightly regulated, the three primary barriers or interfaces that separate the vascular system from the CNS are strictly controlled.^[10] These three barriers include: (i) the arachnoid barrier, (ii) the blood–cerebrospinal fluid barrier (BCSFB), and (iii) the BBB itself.^[23] The makeup and location of these barriers are varied, and are important considerations for the targeting of drugs to the brain (**Figure 2**). The brain itself (as well as the spinal cord) is covered by a triple-layer of connective tissue collectively termed the meninges; separately, the three layers are known as the dura mater, arachnoid mater, and pia mater. Their primary function is to protect the brain from insult or injury, as well as to contain the cerebrospinal fluid (CSF) that bathes the CNS organs. The epithelial cells at the middle layer of the meninges—the arachnoid epithelia—form the arachnoid barrier, and they separate the blood from the subarachnoid CSF. However, this same barrier, situated at the fringes of the brain, forms a relatively avascular membrane that has a total surface area smaller than either the BBB or the BCSFB. This combination causes the arachnoid barrier to play a lesser role in CNS homeostasis, and for that reason it will not be further explored in this Review. The BCSFB, as its name suggests, lies at the interface between the blood and the CSF. Within each hemisphere of the brain exist the lateral ventricles, while the third ventricle is a midline structure in the midbrain, and the fourth ventricle exists in the hindbrain; the primary function of the ventricles is to produce and deliver CSF to the brain and spinal cord. CSF originates from a specialized vascular tissue in each of the lateral ventricles known as the choroid plexus, and it is the epithelial cells that surround this tissue that form the BCSFB.^[5] Since the BCSFB faces a CSF-filled ventricle, and the entire CSF pool in the human brain is turned over every 4–5 hours and thus 5–6 times per day,^[24] it stands to reason that the penetration of

solutes into the brain via this passageway is inefficient at best. Any drug injected into the ventricles is inevitably flushed out of the CNS and back into the blood with little, if any, penetration into the brain. The BBB, on the other hand, has the closest proximity to the neurons, and is therefore considered the most important barrier in preventing unwanted molecules from reaching the brain via its extensive network of blood capillaries.^[10] The BBB is formed by brain microvessel endothelial cells or BMECs (also known as BMVECs and BECs), which separate the blood in the capillaries from the interstitial fluid (ISF) in the brain compartment. Preservation of the BBB is essential for the optimal functioning of the CNS. As discussed previously, the BBB buffers ionic and fluid movements, especially after a meal or physical exercise, to ensure that the ISF provides the most favorable conditions for neuronal function. It also supplies the brain with essential nutrients, mediates the efflux of waste products by the continual turnover of CSF and ISF, separates the neurotransmitters in the CNS from those in the peripheries so that each can act independently, and allows immune surveillance and response with minimal inflammation.^[2, 20] In this regard the BBB acts more like a dynamic interface than a static barrier. The BMECs that make up the BBB do *not* act independently, and they do not maintain permanent, uniform characteristics.^[2] Rather, the various cells of the CNS act in constant cross-talk with each another, and this interdependence regulates the permeability of the BBB as well as the cerebral blood flow (CBF) in the microvessels.^[3, 26] Collectively, these cells, which regulate CBF and BBB function, are known as the neurovascular unit (NVU). The NVU comprises neurons, BMECs, vascular smooth muscle cells (VSMCs), astrocytes, microglia, pericytes, oligodendrocytes, mast cells and even circulating leukocytes, which may influence the permeability of the BBB.^[3, 5, 27, 28] The cross-talk that occurs between each of these cell types is complex, and some of the more intricate communication mechanisms have not yet been elucidated. Nevertheless, the basic functions and actions of most cell types are well established. In the arteries, arterioles and venules of the

brain where smooth muscle exists, VSMCs progressively replace pericytes and serve to modulate the tone of the blood vessels.^[29] Pericytes, which wrap around the BMECs in the capillaries and are enclosed within the endothelial basal lamina, interact with the other cell types to regulate CNS homeostasis, BBB integrity, macrophage activity and CBF modulation.^[9] Pericytes are especially responsible for maintaining the barrier characteristics of the BBB—in adulthood as well as childhood^[5]—and deficiency has been shown to cause a downregulation in the proteins that make up the tight junctions of the BMECs, thus causing BBB breakdown.^[3] Astrocytes, which are star-shaped cells of ectodermal origin, have processes known as perivascular endfeet that are applied to the walls of microvessels.^[30] This close contact with the BMECs enables astrocytes, as well as the pericytes and BMECs themselves, to exert some level of control over the permeability of the BBB.^[28] Additionally, the expression of many water channels (e.g., AQP4) and ion channels (e.g., Kir4.1) on the surface of astrocytes causes them to play an important role in the regulation of water and ion homeostasis.^[2] They also provide nutrients, support and insulation for neurons, and have the ability to secrete cytokines as a sensor of pathological changes.^[9, 21] Microglia are interstitial cells of mesodermal origin that reside in the CNS. They act as macrophages of the CNS, migrating to pathologically affected regions to phagocytose nervous tissue.^[30] Similar to astrocytes, they have the ability to secrete substances such as lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and reactive oxygen species (ROS), which can in turn alter vascular tone and endothelial permeability.^[2, 31, 32] Of the different leukocytes that reside in the body, perivascular macrophages form the primary immune barrier of the CNS in conjunction with local microglia, mast cells and the BBB.^[33] These perivascular macrophages originally start out as circulating monocytes, but frequently migrate across the intact BBB as a result of pathological stimulation and play a key role in mounting innate and adaptive immune responses.^[34] They are especially important since neutrophils are absent

from the CNS, while major histocompatibility complex (MHC) molecules are minimally present.^[10, 30] It can thus be seen that the BBB exhibits dynamic properties due to the simultaneous, coordinated influence of these different cell types (**Figure 3**).

2.2. Physical Characteristics of the BBB

The BBB is a physically imposing gateway that strictly monitors and controls the entry of different substances into the brain.^[19] The BMECs possess unique properties that grant the BBB this characteristic, including (i) tight junctions, (ii) adherens junctions, (iii) apicobasal polarity, and (iv) a luminal surface-bound glycocalyx.

The first and most distinguishable feature of the BBB is the presence of tight junctions. In the absence of endothelial fenestrations, these tight junctions, which constitute a network of strands formed by intramembranous particles, effectively occlude the cleft between BMECs.^[40] This mechanism of sealing the BMECs hinders the paracellular transport of most molecules, forcing them to take other routes to reach the brain.^[37] Indeed, tight junctions are so restrictive that under normal circumstances the effective pore size of BMECs is conjectured to be 1.4–1.8 nm;^[13] Sarin suggests that only particles less than 1 nm in size can be passively transported through the pore.^[37] However, as with other features of the BBB, tight junctions are dynamic structures; they undergo breakdown and reassembly in response to various stimuli, and this hints at the potential for brain drug delivery via manipulation of the BBB.^[41] Tight junctions are located on the apical/luminal region of BMECs, and are formed by an intricate network of parallel, interconnected transmembrane and cytoplasmic proteins. The close association of these proteins confers upon the BBB a high transendothelial electrical resistance (TEER) (1500–2000 $\Omega \text{ cm}^2$), which is hundreds of times greater than the electrical resistance in peripheral capillaries.^[42] Many different transmembrane proteins play a role in the formation and maintenance of tight junctions. The various claudin proteins (claudin 3, 5 and 12) form dimers and bind homotypically to other claudin molecules protruding from

neighboring BMECs, thus forming the primary seal of the tight junction.^[43] It is the expression of these claudins that grants the BBB its high TEER. Occludin is a 60–65 kDa protein that is not essential for tight junction formation, but rather plays a secondary role in regulating and supporting the tight junction. Junctional adhesion molecules (JAM-A, JAM-B and JAM-C) are involved in the formation and maintenance of tight junctions; it is also hypothesized that they may play a role in facilitating leukocyte trafficking.^[43] On the cytoplasmic side of the BMECs the transmembrane proteins connect to a complex array of intracellular proteins (**Figure 4**). These are organized into first-order and second-order adaptor proteins. The first-order adaptor proteins, including Ca²⁺-dependent serine protein kinase (CASK) and the zonula occludens proteins (ZO-1, ZO-2, ZO-3), bind the intracellular domain of the transmembrane proteins and connect them to the various second-order adaptor proteins. In turn, these second-order adaptor proteins (e.g., cingulin) form a scaffold that links the tight junction to the actin/vinculin-based cytoskeleton of the endothelial cell.^[22] Various signaling and regulatory molecules also play a role in controlling the interaction of the tight junction with the intracellular cytoskeleton.^[2, 10] Finally, on the luminal side in the capillaries, BMECs are continuously exposed to a shear stress of 1–10 N cm⁻².^[44] Simulating this level of stress with endothelial cells in vitro increases the expression of tight junction proteins and thereby enhances their barrier properties.^[45] Adherens junctions are located in the basal region of the lateral plasma membrane, adjacent to the tight junctions. Composed of transmembrane glycoproteins, they stabilize cell-to-cell interactions and regulate paracellular permeability by linking the actin filaments in neighboring cells. A large family of calcium-dependent cadherins represents the bulk of these transmembrane glycoproteins, of which cadherin-5, also known as vascular endothelial cadherin (VE-cadherin), is the most important. VE-cadherin is an important determinant of microvascular integrity; overexpression has been shown to inhibit cell proliferation as well as reduce cell permeability and migration. Within the

BMECs, linker molecules including platelet-endothelial cell adhesion molecule (PECAM), the catenins (α -, β -, and γ -catenin), desmoplakin, and p120 catenin mediate the adhesion of transmembrane glycoproteins to the actin cytoskeleton. So vital are the adherens junctions and tight junctions to the BMECs that changes in their phosphorylation state have the potential to weaken their interaction, induce their redistribution, and ultimately lead to increased permeability of the BBB.^[46]

Since the brain is a highly active organ with significant metabolic demands, apicobasal polarity in the CNS is more pronounced than in other systemic regions of the body. This presents itself in several ways, including: (i) differences in composition between apical/luminal and basolateral/abluminal plasma membranes (e.g., membranes can vary in lipid and glycoprotein composition, fluidity, cell surface charge, lipid raft distributions),^[47] (ii) unequal distributions of target receptors between the apical and basolateral membranes (e.g. transferrin receptors are found only on the apical side, therefore only blood-to-brain movement is facilitated), (iii) secretion of specific substances in a polarized manner either from the apical or the basolateral side of BMECs (e.g. platelet-derived growth factor (PDGF) is only released from the basolateral side), and (iv) polarized response to stimuli (e.g. the cytokine IL-6 only facilitates neuroinvasion of HIV-1 if it is directed to the apical membrane).^[45] It has been suggested that the cytoplasmic proteins in both tight junctions and adherens junctions are responsible for initiating and sustaining apicobasal polarity in BMECs.^[2, 10, 45]

Finally, the luminal surface of microvessel endothelia is coated with a carbohydrate-rich covering known as the glycocalyx. This layer is bound to BMECs by glycoproteins and proteoglycans. Heparan sulfate-containing proteoglycans play an especially important role in maintaining and protecting the BBB, by immobilizing potentially neurotoxic molecules whilst mediating cellular uptake of other molecules.^[48] As such, the glycocalyx is important in

regulating leukocyte-endothelial interaction.^[5] Additionally, the presence of sialic acid in the glycocalyx renders it negatively charged, and this is essential for maintaining BBB integrity and function.

2.3. Enzymatic Characteristics of the BBB

Of the many endogenous and exogenous molecules that attempt to penetrate the BBB, some are potentially capable of bypassing the physical barrier imposed by the endothelial cells.^[49] To prevent these molecules from entering the brain parenchyma and interfering with neuronal function, the BBB provides an enzymatic barrier that metabolizes such compounds.

Intracellular enzymes such as monoamine oxidase and cytochromes P450 (CYP450s) can inactivate many toxic and neuroactive substances that enter the BBB. Meanwhile, the plasma membranes of BMECs, pericytes and astrocytes are decorated with a variety of ectoenzymes, including peptidases, nucleotidases, cholinesterases, and others.^[3, 21] The high metabolic activity that occurs at the level of the BBB is responsible for the degradation of most internalized substances.^[9]

2.4. Transport Characteristics of the BBB

Many of the drug delivery mechanisms that are currently being studied make use of the endothelial transport barrier to deliver therapeutics into the CNS. As such, these mechanisms will be discussed in greater detail later on. Nevertheless, some of the same concepts will be highlighted here for the sake of completeness.

The intercellular cleft between endothelial cells is so small that it facilitates very little paracellular transport of molecules. As such, key nutrients that require entry into the brain typically take some sort of transcellular route through the BBB. The first mechanism of BMEC transport is passive diffusion. It has been shown that small lipophilic molecules with molecular weight less than 400–500 Da and less than 9 hydrogen bonds can cross the BBB

freely by simple diffusion.^[50] Indeed, the size requirement is so strict that there exists a difference of eight orders of magnitude between the entry rates of small, lipid-soluble molecules versus large proteins.^[40]

Most nutrients, however, do not fulfill the criteria necessary for passive diffusion. Instead, they use transporters or carriers located on the luminal and abluminal membranes to achieve transit into and out of the brain (**Figure 5**). There are two types of transporters that exist at the BBB: facilitated diffusion transporters, and active transporters.^[2] Facilitated diffusion transporters do not require energy to transport molecules into the brain; rather, they use the concentration gradient generated by the molecules themselves, and this has the overall effect of equilibrating the solute concentrations on either side of the BBB. Active transporters, on the other hand, require energy to move substances against their concentration gradient into (or out of) the brain. The high numbers of mitochondria, which make up for the lack of pinocytotic vesicles at the luminal side of the BBB, facilitate this active movement by supplying the ATP-dependent transporters with energy.^[9]

Two types of action are facilitated at the BMECs: blood-to-brain movement, and brain-to-blood movement. Blood-to-brain movement involves the transport of key nutrients—such as hexoses (glucose, galactose), amino acids and monocarboxylic acids (both charged and uncharged), nucleosides, amines and vitamins—into the brain parenchyma. Since the concentration gradients for these molecules are generally in the direction of blood-to-brain, they utilize facilitated diffusion transporters to cross the BBB. The most notable of these transporters include glucose transporter 1 (GLUT1); monocarboxylate transporter 1 (MCT1); large neutral amino acid transporter 1 (LAT1); and γ^+ , a transporter for cationic amino acids.^[2, 21] Conversely, brain-to-blood movement involves the removal of toxic waste products from the brain. Due to the bidirectional nature of many transporters (including the aforementioned ones), they can be utilized in both blood-to-brain and brain-to-blood

movement. Some ATP-dependent transporters are clustered in polarized locations, including excitatory amino acid transporter 1 (EAAT1) which is found specifically on the abluminal membrane. The BMECs also have a number of ATP-dependent ion pumps located on both membranes that serve to precisely regulate the pH and solute concentration in the endothelium as well as the ISF.^[21]

One of the defining characteristics of the transport barrier is the presence of efflux transporters at both luminal and abluminal membranes (although luminal efflux transporters likely outnumber abluminal transporters).^[21] These efflux transporters play a critical role in preventing many compounds from reaching the brain ISF. Efflux transporters have been discovered for almost every class of substance including ions, amino acids, peptides and cytokines.^[28] The superfamily of ATP-binding cassette (ABC) transporters is primarily responsible for the BBB's ability to efflux such a high proportion of compounds. Among them, P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1), is the most widely known and well characterized. P-gp, although expressed on both sides of the BBB, is largely situated on the luminal membrane, and serves as the primary efflux transporter of many substrates, including a large family of lipid-soluble molecules.^[7] This constitutes a major reason why so many potential therapeutics have historically failed to penetrate the BBB.^[28] Other examples of ABC efflux transporters include the multidrug resistance-associated proteins (MRPs)—specifically MRP1, MRP2, MRP4, and MRP5—and breast cancer related protein (BCRP).^[21] BCRP, whose in vivo function is likely modulated by P-gp, is the most abundant of ABC transporters in the human brain, and has been identified as a key contributor to BBB selectivity as a result of its ability to efflux various xenobiotics from the brain.^[52, 53] Collectively, the various luminal and abluminal transporters are believed to work together to prevent foreign compounds from penetrating the BBB.^[21, 53]

Although the use of carriers/transporters is ideal for small nutrient molecules, larger

macromolecules are unable to make use of the same pathways. The sheer size of these compounds (relative to small nutrient molecules) prevents them from being taken up by the transporter proteins, and so an alternative mechanism—receptor-mediated transport—is employed instead. Various receptors are expressed on the BMECs, and the binding of a specific ligand to its BBB receptor triggers uptake of the compound into a vesicle that subsequently pinches off from the membrane and undergoes endocytosis or transcytosis. Although endocytosis arguably constitutes the first step in the process of transcytosis, the key difference between the two processes is that endocytosis involves trafficking a compound into the cytoplasm of the endothelial cell, whereas transcytosis involves trafficking the compound through both luminal and abluminal membranes. Various factors, including the intravesicular environment and the type of receptor itself, determine whether endocytosis or transcytosis occurs.^[54] Despite the fact that a lower degree of endocytosis and transcytosis occurs in BMECs compared to peripheral endothelial cells, these processes are still important for the delivery of many different growth factors, enzymes and plasma proteins into the brain.^[2] Some of the most well-studied receptors, especially in the context of drug delivery, include the insulin receptor (IR), which regulates glucose homeostasis; transferrin receptor (TfR), which mediates the cellular uptake of iron bound to transferrin molecules; lipoprotein receptors (LDLR (low density lipoprotein receptor), LRP1 and LRP2 (low density lipoprotein receptor-related protein 1 and 2)), which are multifunctional, multi-ligand scavenger and signaling receptors; and diphtheria toxin receptor (DT_R), which uniquely has no known endogenous ligand,^[55] but which can mediate the entry of diphtheria toxin especially under inflammatory conditions when the receptor is upregulated.^[56, 57] The heterogeneous and polarized distribution of receptors along the BBB ensures that macromolecules can undergo targeted transport to specific areas of the brain where they are required. For example, TfRs are only found on luminal membranes, and so facilitate only blood-to-brain transport,^[28, 45] while

the LRP receptors are highly expressed in the cerebellum, cortex, hippocampus and brainstem.^[56, 58] This heterogeneity is of interest, as it can potentially be harnessed for the safe and efficacious delivery of therapeutics to diseased regions of the brain.

2.5. Immunological Characteristics of the BBB

The brain's immunological properties are unique and somewhat different from other regions of the body. It is not an immune-privileged organ, but rather a site of selective and modified immune reactivity.^[30] No lymphatic vessels have been identified in CNS tissue, and even though 50% of CSF drains into the cervical lymph node (the other 50% returns to venous circulation), there is still a relative lack of parenchymal lymphatic drainage compared to other, non-CNS organs.^[30] In addition, the mature CNS appears to lack endogenous antigen-presenting cells (APCs) that typically serve to mediate cellular immune responses, for example by recognizing, phagocytosing, processing and presenting foreign antigens to T cells.^[59, 60] This process of antigen recognition and binding is mediated by cell-surface proteins known as MHCs. Under normal conditions, MHC class II molecules are expressed in low levels in the human brain and are restricted to reactive microglia and phagocytic macrophages—cells that have limited capacity for antigen presentation to naïve cells.^[30, 61] Few leukocytes reside in the CNS unless inflammation occurs; neutrophils, as mentioned previously, are virtually non-existent. Yet of the endogenous leukocytes that are present, the majority is made up of T cells (80% of the leukocyte population in the CNS versus 45% of the leukocyte population in the peripheries).^[62] These T cells are activated upon demand when immune reactivity in the brain is temporarily induced. Ultimately, the immune barrier of the BBB is upheld by close interactions between BMECs, perivascular macrophages, mast cells, microglia and T cells (**Figure 6**).

Chemokines (a subset of cytokines) serve as important chemo-attractants that stimulate the entry of leukocytes into the CNS, albeit slower than in the peripheries due to the

restrictive nature of the BMECs. It has been shown that an injection of neurotoxin into the CNS can elicit an immediate reaction from resident microglia, with a delayed reaction from circulating monocytes since they are slow to differentiate into macrophages and cross the BBB.^[64] Finally, it has been proposed that after initial T cell stimulation in the CNS, the final immune reaction is not actually amplified in the CNS itself.^[30, 65] Rather, due to the tightly regulated nature of the BBB, the amplification takes place in secondary lymphoid organs before being restimulated in the CNS by interactions between memory T cells and APCs.^[30, 65]

3. Overview of BBB Pathology

The physiology of the BBB undergoes significant pathological changes during the course of a neurological disease. Although the nature and extent of such changes vary from condition to condition, one key commonality is the breakdown and improper functioning of the NVU. In the context of CNS drug delivery, it is important to identify the etiology and molecular basis of a disease if effective therapeutic strategies are to be developed. This section will provide a general overview of NVU pathology, as well as a brief discussion of four debilitating neurological conditions—AD, MS, PD and stroke.

3.1. The Significance of NVU Interactions in BBB Pathology

The successful day-to-day functioning of the CNS hinges upon precise autocrine and paracrine communications between cells of the NVU.^[28] The pericytes, BMECs, neurons and glial cells require stimulation by specific neuroimmune agents in order to regulate blood flow, microvascular permeability, angiogenesis, neurogenesis, cell matrix interactions and neurotransmitter levels.^[21] Whilst many of these agents serve to improve BBB permeability and function, some are capable of doing the exact opposite.^[2] And although the etiology of different neurodegenerative diseases tends to vary from condition to condition, one of the

fundamental underlying pathological hallmarks is disruption of the BBB (**Figure 7**).^[40] The cells of the NVU are extremely sensitive to a number of different substances, including cytokines (e.g. IL-1 β , IL-6, TNF- α , interferon- γ (IFN- γ)), lipid mediators (e.g. prostaglandins), oxidative compounds (ROS, free radical nitrogen oxide (NO), peroxide (H₂O₂)), vasogenic agents (e.g. histamine, vascular endothelial growth factor (VEGF)), infective agents (e.g. bacteria, viruses, fungal pathogens), and other endogenous stimuli (e.g. extracellular K⁺, intracellular Ca²⁺).^[46] Many of these substances are released under pathological conditions, and their effects tend to be localized and transient. As a result, there is a direct correlation between neuroinflammation and levels of these substances in the brain.^[2] Some of the steps that follow include alteration or breakdown of the physical, transport, and immune barriers.

3.1.1. Breakdown of the Physical Barrier

The accumulated expression of agrin (a heparan sulfate-containing proteoglycan) on the basal lamina of BMECs is important for the integrity of the BBB. The basal lamina, composed of extracellular matrix (ECM) structural proteins, forms a mesh around the BBB that blocks the passage of macromolecules into the brain.^[47] Neuroinflammation, however, results in the loss of agrin from the abluminal surface of BMECs. Depending on the type of condition, this may contribute to BBB damage and brain edema caused by the unregulated redistribution of astrocytic aquaporin channels (needed for the regulation of water levels).^[66] Pericyte deficiency can downregulate the expression of tight junction proteins including claudin 5, occludin and ZO-1.^[67] In many neurodegenerative disorders, matrix metalloproteinases (MMPs)—which are endopeptidases capable of degrading ECM proteins and bioactive molecules—are known to attack occludin as well as basal lamina proteins such as fibronectin, laminin and heparan sulfate.^[21] The inevitable weakening of tight junction and adherens

junction integrity leads to the loss of apicobasal polarity, and this may play a role in attracting immune cells to invade the now-vulnerable CNS.^[45]

3.1.2. Alteration/Breakdown of the Transport Barrier

The normal pattern of transporter expression is often altered under the influence of neuroimmune modulators, and the level of such expression can vary from condition to condition. GLUT1, for example, is upregulated in starvation and hypoxia as an adaptive mechanism.^[68] P-gp is upregulated in epilepsy and after oxidative stress, but downregulated in AD and PD.^[2, 69-72] Spinal cord injury hinders the transport rates of neuroinflammatory molecules such as TNF, pituitary adenylate cyclase-activating peptide (PACAP) and leukemia inhibitory factor 50 (LIF-50), whereas the inverse is observed in stroke patients.^[28] The various receptors, including T_fR, IR, LRP, LDLR, and DT_R, undergo changes in their expression during neurodegenerative disease, and such changes are often clustered in specific regions of the brain. DT_R is especially prone to upregulation in many inflammatory conditions.^[56] Indeed, many agents can modulate transporter function during disease. And although some of the effects are compensatory—and therefore positive—often the action of these agents results in further BBB dysfunction.

3.1.3. Invasion of the Immune Barrier

As an extension to transport barrier alteration, increased leukocyte trafficking into the CNS is generally regarded as a universal symptom of neuroinflammation (**Figure 8**). The secretion of inflammatory mediators, in addition to transiently opening the BBB, also appears to upregulate the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1).^[74] The result is increased transendothelial migration of immune cells.^[74] Due to the heterogeneous population of these cells, some leukocytes (e.g. regulatory T (T-reg) cells and myeloid suppressor cells (MSCs)) may actually exert immunosuppressive effects.^[75] However, others propagate the disruption of the BBB by initiating and amplifying

inflammatory effects (**Figure 9**).^[77] Indeed, there is evidence to suggest that immune cell invasion of the CNS is directly correlated with clinical symptoms; this is true for glioblastoma multiforme (GBM), MS, encephalitis, meningitis and HIV-1, among other conditions.^[21, 76] In the case of HIV-1, the trafficking of infected monocytes through the BBB is the primary disease mechanism. Also known as the “Trojan Horse” method, this transport route enables pathogens to enter the CNS disguised, and therefore undetected, by using immune cells as vectors.^[21]

On the basis of preclinical and clinical studies, it is proposed that many neurological disorders share a common set of traits known as the “vasculo-neuronal-inflammatory” triad.^[78] As a result of the aforementioned processes, most significant pathologies of the CNS involve: (i) vascular damage, (ii) neuronal injury and neurodegeneration, and (iii) neuroinflammation.^[78] However, the intricate way in which these complications coalesce to cause specific neurological diseases is not yet fully understood.^[21] Further work in uncovering these mechanisms will better inform efforts in designing drug systems against specific neurological disease targets.

3.2. Alzheimer’s Disease (AD)

As the global population continues to age, the prevalence of neurodegenerative disease is on the rise. It is forecasted that at the current rate, 1 in 85 persons worldwide will be living with AD by 2050.^[79] As a result, the development of new interventions that can delay, halt or reverse disease onset and progression is an area of research receiving intense interest. AD, which constitutes the most prevalent form of dementia (around 70% of dementia cases^[80]), is a chronic and progressive brain disease that leads to impaired memory, and changes in thinking and behavior. Second to AD is vascular dementia (VaD), which makes up around 15% of dementia cases and is characterized by a greater degree of clinical and pathological heterogeneity.^[80, 81] Although historically these two diseases have been separately and

distinctly identified, more recently it has been hypothesized that AD and VaD may lie on a disease continuum, where polymorphisms contribute to the differences in cases.^[7, 82]

The two hallmarks of AD are: (i) the buildup of cortical and cerebrovascular deposits of amyloid- β ($A\beta$) peptide, and (ii) the accumulation of unnatural levels of hyperphosphorylated, microtubule-associated tau protein (**Figure 10**).^[83-85]

Although $A\beta$ uptake and clearance mechanisms in both normal and diseased states have been well studied, there still exist some gaps in our understanding of the fundamental biology. Under normal physiological conditions, $A\beta$, which is formed from cleavage of the $A\beta$ precursor protein (APP), is produced in the brain (CSF $A\beta_{1-42}$ levels are more than ten times that of plasma levels).^[87, 88] It is also possible that some $A\beta$ is taken up into the brain from the periphery by the receptor for advanced glycation end products (RAGE).^[89, 90] However, it is suggested that the failure of $A\beta$ clearance from the brain has more impact on its accumulation rather than endogenous production or peripheral uptake. By using metabolic labeling of human AD patients and controls, Mawuenyega et al. demonstrated that $A\beta$ is produced at similar rates in AD subjects and controls, however, the clearance of $A\beta$ from the brain is retarded in people with AD.^[91] One proposed clearance mechanism is the proteolytic degradation of $A\beta$.^[92] Several $A\beta$ -degrading enzymes within the CNS have been identified, including neprilysin (NEP), insulin-degrading enzyme (IDE), plasmin, endothelin-converting enzymes 1 and 2 (ECE-1, ECE-2), angiotensin-converting enzyme (ACE), and the MMPs (MMP-2, MMP-3, MMP-9).^[93] Of these enzymes, NEP is considered one of the most important for the control of cerebral $A\beta$ levels.^[92, 93] The correlation between levels of NEP and enzymatic activity has been demonstrated in experiments conducted by Iwata et al. and Marr et al.^[94, 95] Using radiolabelled $A\beta$, Iwata et al. showed that NEP was primarily responsible for degrading $A\beta_{42}$ and that subsequent NEP inhibition caused dramatic elevations of endogenous $A\beta$ resulting in plaque deposition.^[95] Marr supported this finding by

observing that NEP overexpression resulted in significant reductions in A β plaque deposition in APP-transgenic mice.^[92, 94] Further mechanisms of A β clearance are suggested to involve the bulk flow of interstitial fluid, as well as transport across the BBB via LRP1.^[3, 96]

Under normal physiological circumstances for humans, both A β synthesis and clearance are understood to be well-regulated processes.^[97] In AD, however, these processes are disrupted, and the net result is increased production, reduced degradation, and impaired clearance of A β .^[7, 91] One study found that the accumulation of low levels of copper in the brain capillaries and the parenchyma could be directly correlated to increased brain A β levels, and thus the development of AD in mouse models.^[96] This finding has been confirmed by separate in vitro and in vivo studies, suggesting that A β aggregation and neurological toxicity can be caused by abnormal interactions with metal ions in the neocortex, especially zinc, copper and iron.^[98-101] Cherny et al. showed that by treating AD transgenic mice with a copper-zinc chelator, levels of neocortical A β accumulation could be rapidly and dramatically reduced, thereby indicating a potential disease-targeting application of metal-attenuating compounds.^[102]

The most common (but now increasingly disputed) explanation for the pathogenesis of AD revolves around the A β protein. The neurovascular hypothesis or amyloid hypothesis proposes that A β accumulation *initiates* the cascade of events leading to neuronal injury and thus AD progression.^[28] A growing body of evidence, however, suggests that this view might be incomplete.^[83, 103, 104] The neurovascular hypothesis, in itself, does not account for the vascular factors that also underpin the disease.^[105] BBB disruption, decreased CBF, increased capillary tortuosity, increased IgG antibody trafficking, and neurotoxic secretions from BMECs have all been observed to some extent in AD cases.^[106] In fact, these vascular factors are so critical that reduced CBF is considered one of the earliest pathological signs of disease progression. It is commonly observed in at-risk elderly individuals, and its onset precedes any

signs of cognitive decline, brain atrophy and A β accumulation.^[105, 107] As such, an alternative theory has been set forth.

The two-hit vascular hypothesis suggests that AD results from a dual-stage process. The first “hit” (e.g., potentially triggered by hypertension, diabetes, stroke) involves damage to the brain microcirculation, which results in BBB dysfunction and oligemia (i.e. blood volume deficiency due to reduced CBF). In addition to the buildup of neurotoxic substances (many cytokines are found in elevated levels in AD),^[108] BBB dysfunction creates a hindrance to A β clearance, whilst oligemia leads to increased A β production. This untoward combination results in the second “hit”, which is the accumulation of A β peptide and tau protein in the brain.^[3] Collectively, these two hits are suggested to contribute to the pathologies and symptoms associated with AD. In a recent study, however, Keren-Shaul et al. identified a unique type of microglial cell—a result of immune heterogeneity—that may play an endogenous role in halting the progression of neurodegeneration.^[109] These microglia, known as disease-associated microglia (DAM), were observed to localize near sites of AD pathology and exhibit enhanced phagocytic activity towards A β plaques.^[109]

In AD mouse models, targeted and temporary disruption of the BBB by scanning ultrasound has also been reported to yield some physiological benefit. SUS was shown to reduce amyloid plaque formation and improve memory performance in 75% of mice treated with this procedure.^[110] This provides initial evidence that temporary opening of the BBB may not necessarily be detrimental in AD, but may instead facilitate the clearance of A β from the brain.

Finally, AD shows heterogeneous pathology. In a large study of post mortem cases, only 55% of Alzheimer’s cases had amyloid and tangle pathology alone, while the remainder had infarct and Lewy body pathology,^[111] including 37% of cases that had vascular pathology. The degree of cerebrovascular disease tends to vary, since abnormalities of the cerebral

vasculature may develop due to any number of underlying causes.^[7] As a result, it is distinctly possible that BBB dysfunction is an exacerbating feature in a subgroup of AD cases.

3.3. Multiple Sclerosis (MS)

MS is a chronic neuroinflammatory disease characterized most strikingly by the demyelination of neurons and the CNS invasion of immune cells, especially lymphocytes and macrophages (**Figure 11**).^[28] The cause of the disease has not yet been fully characterized, but is thought to involve an interplay between environmental factors (especially low levels of vitamin D), smoking, Epstein-Barr virus (EBV) (a herpes virus that causes mononucleosis/glandular fever), and genetic factors (including risky gene variants, such as the widely known HLA-DR2 allele).^[112] It is estimated that almost 2.3 million people worldwide suffer from the disease, mostly between the ages of 20 and 40.^[112] There are three types of MS: (i) relapse–remitting MS (RRMS), which produces attacks followed by periods of remission, (ii) primary progressive MS (PPMS), which produces steadily-worsening symptoms without remission, and (iii) secondary progressive MS (SPMS), wherein RRMS evolves over time into a progressive disability without remission.^[113] Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics some of the clinical and histopathological characteristics of MS.^[30] It is used as the basis for studying the pathophysiology of MS, as well as investigating the pre-clinical efficacy of therapeutic strategies to combat the disease. EAE has its own limitations, however, in that some of its underlying disease mechanisms may not accurately reflect those that occur in MS—for example, there is evidence certain cytokines playing divergent roles in each condition.^[114] Understanding the key differences between EAE and MS is essential for the rational design of therapies that effectively combat demyelinating disease.

MS principally affects the white matter of the brain, spinal cord and optic nerve.^[116] It is suggested that peripheral signals act on cells of the NVU, inducing them to secrete substances

that act on BMECs and increase endothelial permeability.^[117] Expression of IL-1 β is suggested to have some association with the selective upregulation of MMP-9,^[118] which is known to cleave tight junction and adherens junction proteins.^[119] Cytokines IL-17 and IL-22 decrease expression of occludin and ZO-1, whilst IFN- γ and TNF- α reversibly induce the loss of VE-cadherin and the redistribution of other molecules.^[120] In addition to breakdown of the junctional complexes, alteration of the basal lamina is also induced. Dystroglycan, which is a basal lamina protein, may be cleaved by MMP-2 and MMP-9,^[121] while other basal lamina components (e.g. laminin-8 and laminin-10 isoforms) may be overexpressed, leading to increased immune cell infiltration.^[7, 122] Meanwhile, the overexpression of adhesion molecules (e.g. VCAMs, ICAMs, E-selectin, ALCAM) and chemokines induces immune cell invasion of the CNS via diapedesis across the BBB.^[123] Although some of the leukocytes that enter exert a regulatory, immunosuppressive effect on the inflammatory process, many (especially CD4+ T cells) play a crucial role in the sustained progression of MS.^[124] These release neuroinflammatory molecules that break down the myelin sheath insulating axons in the parenchyma. With no protective coating, exposed fibers are degraded and electrical signals can no longer be propagated effectively. The symptoms that arise depend on the level of damage sustained by the axons, as well as the location where such damage occurs.^[113]

3.4. Parkinson's Disease (PD)

PD is a chronic, progressive neurodegenerative disorder that presents with classic motor impairments including tremors, rigidity, slow movement (bradykinesia), poor balance, and difficulty walking (Parkinsonian gait), and these symptoms tend to be attenuated by dopamine therapies.^[21] Non-motor symptoms include dementia, orthostatic hypotension, constipation, REM sleep behavior disorder, depression and impotence. Similar to other CNS diseases, dysfunction of the BBB has been observed in PD (**Figure 12**), although it still remains to be determined whether its role is causative or simply a manifestation of the disease.

Unsurprisingly, BBB disruption is triggered by the release of inflammatory mediators that remodel the junctional proteins and cause transient “opening” of the endothelial cells.^[126] In addition, P-gp is significantly downregulated, thus preventing the efflux of many toxic substrates from the CNS.^[70] As a result, dopaminergic neurons (dopamine-producing nerve cells) typically located in the substantia nigra and the locus coeruleus are destroyed (one of the key metabolizing agents is monoamine oxidase).^[127-129] Degeneration may also take place in the dorsal motor nucleus of the vagus nerve, located in the medulla. By the time PD patients experience the onset of clinical symptoms they have typically lost more than 80% of dopaminergic neurons.^[21] Since dopamine is an essential CNS neurotransmitter and peripheral chemical messenger, its depletion prevents the motor nerves from performing their function properly—namely, controlling movement and coordination.

3.5. Stroke

As of 2015, stroke was ranked among the leading causes of years of life lost in most regions around the world, accounting for more than 11% of total deaths globally.^[130] It is characterized by the appearance of neurological dysfunction (either local or widespread) as a result of vascular causes. There are two types: ischemic stroke, which occurs due to vessel occlusion, and hemorrhagic stroke, which occurs due to bleeding (**Figure 13**). Cerebral ischemia, accounting for 80–85% of stroke cases,^[7] will primarily be explored in this Review.

The onset of stroke, per se, actually occurs *secondary* to the occlusion of a cerebral vessel by a clot (thrombus or embolus). Vascular obstruction causes a drop in CBF downstream, and the resulting lack of blood flow quickly initiates an ischemic cascade that involves neuronal cell death and neuroinflammation.^[132, 133] Neurons lack self-sufficient energy stores, and are thus heavily reliant on the cerebral vasculature for the provision of oxygen and glucose. When an ischemic event stifles blood flow to a specific region of the brain, neurons in that area depolarize and release glutamate (an excitatory neurotransmitter),

which binds to ionotropic receptors (receptor proteins that also contain ion channels) and triggers the toxic entry of calcium into the neurons. The result is excitotoxic cell death.^[133]

Neuroinflammation is believed to be strongly linked to this neuronal injury, as well as to the acute and delayed downstream effects of brain infarction, and a key hallmark in the condition's pathophysiology is BBB disruption.^[132, 134]

There are generally two phases of BBB disturbance after cerebral ischemia: (i) an initial, acute disruption of the BBB 3–5 hours following the ischemic event; followed by (ii) a widespread increase in BBB permeability at 48 hours, likely as a result of cerebral ischemia reperfusion, accompanied by further expansion of the cerebral infarct size.^[135] As is expected, there are various factors that contribute to this (**Figure 14**). Several MMPs are overexpressed after ischemic stroke. For example, MMP-2 and MMP-9 have been observed to degrade claudin-5 in rat models of ischemia, and the concerted action of these enzymes have been shown to contribute to a noticeable absence of tight junction proteins from the brain endothelium.^[136] MMP-9 could also play a role in cleaving ZO-1 and degrading basal lamina proteins such as collagen IV, fibronectin and laminin.^[7] Hypoxia can result in ROS being produced, causing further breakdown of endothelial junctions.^[137] Transport of neuroinflammatory molecules TNF- α and PACAP is upregulated.^[28] AQP4 is increasingly expressed on BMECs, resulting in the shift of fluids from the intravascular space to the ischemic brain. Edema ensues, and this is a key indicator of BBB disruption.^[138, 139] Surprisingly, P-gp is upregulated during hypoxia after focal cerebral ischemia, probably as a recovery mechanism.^[140] Indeed, in the early stages after an ischemic event, inhibition of BBB opening can reduce brain damage caused by the drop in CBF.^[13] Also significant is the observation that astrocytes secrete transforming growth factor- β (TGF- β), which acts to downregulate endothelial expression of tissue plasminogen activator (tPa) and thrombomodulin (TM).^[141] tPa is a fibrinolytic protease that converts plasminogen to its

active form, plasmin; plasmin is the major enzyme responsible for clot breakdown. Meanwhile, TM is an integral membrane protein that serves as a cofactor for thrombin. After binding to TM, thrombin is converted to an anticoagulant enzyme that also plays a role in the removal of the vascular obstruction. Thus, inhibition of tPa and TM may perpetuate the disease mechanisms described above.^[141] Indeed, the pathophysiological mechanisms involved in the development of stroke are already quite well characterized.^[7] Notwithstanding this, attempts to develop suitable therapeutics combating the disorder have been only minimally successful. Recombinant tPa is currently used as a clinical treatment for stroke, despite its modest efficacy, short therapeutic window and some deleterious effects.^[143-145] In light of this, new effective methods of drug delivery through the BBB may help to overcome these challenges.

4. Pathways to Access the Brain

Drug delivery to the brain can be categorized into two main areas: bypassing the BBB, and crossing the BBB. Section 4.1 will provide a concise overview of the various strategies that can be used to deliver therapeutics to the CNS without having to build in BBB-crossing functionality (**Figure 15**). There are also several endogenous pathways that can be exploited with the purpose of facilitating drug passage across the BBB, and these will be discussed in Section 4.2.

4.1. Bypassing the BBB

4.1.1. Intracerebroventricular (ICV)

ICV administration involves the penetration of the skull and the direct injection of a drug into the CSF-filled lateral ventricle of the brain. The drug is introduced via an outlet catheter leading from an implantable reservoir, or alternatively via a pump. Of the two methods, the pump is used more readily since it is able to achieve a more continuous, elevated

concentration of drug in the CSF.^[14] There are several benefits associated with this method of bypassing the BBB, including diminished systemic toxicity, no drug metabolism in blood serum, and no opsonization by serum proteins.^[14] Nevertheless, ICV administration also carries some significant drawbacks and risks. As mentioned previously, the entire CSF pool in the human brain is turned over every 4–5 hours.^[24] It exits via bulk flow, and is absorbed into the bloodstream by arachnoid villi at the superior sagittal sinus.^[146] Meanwhile, any drug that is infused via ICV can only penetrate the brain parenchyma by the slow process of diffusion. This involves crossing the BCSFB and navigating the extracellular space of the parenchyma, which is defined by its high tortuosity and restricted pore size.^[147] Since the rate of CSF bulk flow is orders of magnitude greater than diffusion, drug often exits the ventricles faster than it can diffuse into the brain. In fact, the difference is so great that in many cases, 98–99% of the original drug concentration in the CSF is lost at a distance of only 1–2 mm from the parenchymal surface.^[148] It is suggested that continuous ICV infusions (Figure 15A) may prove more efficacious than bolus injections, allowing for greater drug dispersion, but the clinical effectiveness of this treatment still has yet to be fully validated.^[9] Since the procedure is invasive, other associated risks that have to be considered include infections and increased intracranial pressure due to fluid injection.^[14]

4.1.2. Intracerebral/Intraparenchymal

Intracerebral or intraparenchymal administration involves the delivery of drugs directly to the brain parenchyma via an implant or injection (Figure 15C).^[14] Similar to ICV, drug movement occurs solely due to passive diffusion through the ISF. This process is so slow and limited that drug molecules experience little penetration into surrounding tissues, spreading no further than ca. 2 mm from the injection site as a result of diffusive processes.^[149, 150] Fung et al. investigated the drug distribution profile for chemotherapeutic agents released from polymer implants in the rat brain, and observed, over a 30-day period, the drug penetration

distance—defined as the distance removed from the injection site where drug concentration levels dropped to 10% of the maximum.^[151] The penetration distance was determined to be 5 mm at day 1, before dropping to 1 mm by day 3.^[151] Furthermore, the spike at day 1 was determined to be due to extracellular fluid convection arising from temporary vasogenic edema—once this subsided, the drug penetration distance decreased accordingly. Considering tumors (as well as other CNS pathologies) can spread significant distances from sites of surgical resection and drug treatment (~1 cm), the limited penetration associated with intraparenchymal drug administration can hinder the efficacy of the therapy.^[151]

4.1.3. Convection-Enhanced Delivery (CED)

CED is a therapeutic strategy that involves minimally invasive surgical exposure of the brain, followed by the placement of small diameter catheters into the parenchymal interstitial space.^[14] A drug solution is then infused via this apparatus (Figure 15B). However, instead of relying on diffusion as the sole means of drug distribution, a positive pressure gradient is applied to the solution by an external source such as a pump. This causes fluid convection in addition to diffusion, and ultimately results in a greater volume of distribution than that achieved under intracerebral administration (**Figure 16**). Smaller molecules tend to exhibit greater distribution volumes than larger molecules or particles.^[152]

The efficacy of CED has been validated in animal models bearing tumor stem cells. GBM is a grade IV malignant glioma (brain tumor) that usually results in death; the median survival rate is less than 15 months.^[154] Conventional chemotherapies fail because, even if they manage to penetrate the brain parenchyma, they do not reach the brain cancer stem cells (BCSCs) that are responsible for tumor development.^[155, 156] To enhance the depth of penetration of locally delivered therapeutics, Zhou et al. loaded BCSC-combating agents into NPs and administered them via CED into rats and pigs. The result was that these NPs were able to traverse white matter tracts in the brain and reach the corpus callosum. One of the

tested drugs was even able to significantly increase survival rates in rats bearing BCSC-derived xenografts.^[155]

It is evident that CED's locoregional distribution properties render it a more clinically promising strategy than many other neurosurgical measures.^[153] Nevertheless, it is not without risks. Invasive procedures come with the risk of infection. In addition, high pressures associated with convective flow can cause the unwanted diversion of fluid into more sensitive and less flow-resistant regions such as the subarachnoid space. Poor catheter placement can lead to tissue injury and air bubbles.^[14] Various microfluidic devices have been developed to reduce the chances of neurological injury, but further comprehensive studies are required to confirm the safe and valid use of CED in clinical practice.^[157]

4.1.4. Intrathecal

Of the various neurosurgical procedures that have been shown to bypass the BBB, intrathecal administration is arguably one of the least invasive. Therapeutic agents are injected into the subarachnoid space of the spinal cord, usually via lumbar puncture, and are delivered to the CNS parenchyma through the CSF (Figure 15D).^[9] However, there is evidence to suggest that the flow of CSF and parenchymal ISF is actually in opposition to the desired direction of drug transport.^[158] Nevertheless, it is believed that leptomeningeal transport, which is dependent upon the location and volume of drug administration, is primarily responsible for overcoming these fluxes and successfully delivering therapeutics to the parenchyma.

Leptomeningeal transport consists of four consecutive processes, including: (i) convective movement of drug solutes through the CSF, driven by pulsatile mixing; (ii) active pumping of CSF into perivascular spaces; (iii) movement of solute into the parenchyma; and (iv) neuronal uptake and axonal transport.^[9, 158] Studies suggest that intrathecal administration may be a feasible drug targeting strategy, since macromolecules and NPs can be delivered in biologically significant amounts (>1% of injected dose).^[158] The strategy is currently being

tested in human trials with patients suffering from MS and spinal cord injury, and initial phase I trials using antibodies have revealed no major side effects.^[7] In a mouse model of EAE (to simulate human MS), protein antibody-derived therapeutics were delivered to the CNS via both intrathecal and intracerebral routes.^[159] Results conclusively showed that intrathecal administration produced more potent EAE suppressive effects than intracerebral administration of the same drug.^[159] However, potential risks associated with intrathecal administration include infection and dose-dependent immunogenicity, the latter of which is not well understood.^[9]

4.1.5. Intratympanic

In the context of CNS therapeutics, inner ear drug delivery is a seldom-explored avenue, despite its potential viability.^[160-162] The anatomy of the inner ear is so closely linked with the CNS that methods have been developed to exploit this pathway without the need to factor in interactions with the BBB. Labyrinthine perilymph is an extracellular fluid located within the cochlea of the inner ear, which has ionic composition comparable to that of blood plasma and CSF.^[163] The perilymph reportedly communicates with the CSF of the subarachnoid space through the cochlear aqueduct, also known as the perilymphatic duct. Although the amount of fluid transfer may be limited, it is suggested that CSF may reach and mix with the perilymph as far as the scala tympani (one of the three compartments of the cochlea).^[164] Within this framework, intratympanic delivery most commonly involves the injection of a drug into the middle ear cavity (Figure 15F), followed by pinocytotic transport of the drug through the round window membrane (RWM)—the RWM forms the most important interface between the middle ear and inner ear, and consists of three layers located at the basal end of the scala tympani (an outer epithelium that faces the middle ear; a middle layer; and an inner epithelium that borders the scala tympani).^[165, 166] Studies suggest that macromolecular agents up to 1 μm in size can traverse the RWM, reach the perilymph and then move through the

CSF to reach the brain, bypassing the blood–labyrinthine barrier (BLB) in the process (the BLB is similar to the BBB).^[166, 167] Zhang et al. conducted *in vivo* experiments, administering 5 different therapeutic agents—all of which were incorporated into poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) (see Section 5.3.1)—via the intratympanic route.^[165] These experiments revealed that the CSF and brain drug distribution profiles were most optimal when drugs were incorporated in NPs and administered via the intratympanic route than when administered intravenously (CSF concentrations of 2 of the therapeutic agents—salvianolic acid B and tanshinone IIA—were found to be more than 3.5 times higher after intratympanic administration when compared with intravenous administration).^[165] Cytotoxicity profiles were also favorable. Measurement of nitric oxide synthase (NOS), malondialdehyde (MDA) and superoxide dismutase (SOD) levels after intratympanic delivery (all of which are biomarkers of cytotoxicity) revealed no signs of cochlear injury.^[165] Integration of the pharmacodynamic and pharmacokinetic profiles ultimately led to the conclusion that intratympanic delivery of NPs was a more effective approach to treating neurodegenerative disease than systemic administration.^[165]

4.1.6. Intranasal

Intranasal administration is another method of bypassing the BBB. However, in contrast to most other approaches discussed in this section it is a noninvasive method that does not require an injection. Therapeutic agents are sprayed high up in the nasal cavity, at the level of the cribriform plate, to facilitate absorption by the olfactory and respiratory mucosa (Figure 15E).^[168] There are three pathways by which drugs are suggested to reach the brain's entry points located at the olfactory bulb and brainstem.

(1) Extracellular diffusion (and convection): after moving either paracellularly or transcellularly through the olfactory and respiratory epithelia, drugs may undergo extracellular transport (via diffusion and/or convection) through perineural, perivascular or

lymphatic compartments surrounding the olfactory and trigeminal nerves until they reach the olfactory bulb or brainstem.^[169] This process lacks targeting specificity and is arguably less desirable than the other two pathways, since drugs could end up being absorbed into blood vessels or lymphatic vessels and entering the body's systemic circulation as a consequence.^[169]

(2) Intra-neuronal transport via the olfactory sensory neuron (OSN): drugs may be taken up into the OSN by endocytosis, and subsequently transported from the level of the olfactory epithelia to the olfactory bulb via anterograde axonal transport (movement away from the neuronal cell body and towards the synapse).^[169]

(3) Intra-neuronal transport via the trigeminal nerve: in this pathway, drugs may undergo endocytosis into peripheral trigeminal nerve processes located near the respiratory epithelial surface, at the level of either the maxillary sinus, middle nasal concha, or choana.^[169, 170] From there, intracellular axonal transport may subsequently enable drugs to reach the brainstem and therefore access other regions of the CNS.^[48, 169]

In both of the aforementioned intra-neuronal pathways, interneuron translocation is also proposed to occur, whereby drugs are transferred from first-order, peripheral neurons (OSNs and trigeminal nerves) to second-order neurons whose diameter reportedly measures between 100–330 nm; these second-order neurons ultimately synapse onto target sites within the CNS.^[48, 169]

Although distribution patterns vary from drug to drug, there are several benefits associated with intranasal administration, including rapid absorption, rapid onset, avoidance of hepatic first-pass metabolism, noninvasiveness, and patient comfort and compliance.^[13] The use of NPs can enhance this effect since it can enable higher payload capacities, selective targeting, controlled release and increased drug retention on the nasal mucosa.^[171] In experiments conducted by Xia et al., surface-modified NPs were found to yield a greater brain

drug concentration when delivered by the intranasal route, than when delivered by conventional oral and intravenous routes.^[170] It was also determined that particles smaller than 100 nm have a higher mucosal transport, potentially because this size facilitates their uptake into the endocytotic pathways of the OSN and trigeminal nerve.^[170] Such findings were validated by Kanazawa et al., who found that small (100 nm) polymeric micelles were able to deliver coumarin, a model chemical, to C6 glioma cells in the rat brain more effectively via intranasal administration than via intravenous administration. A biodistribution analysis revealed that the intranasal delivery of these micelles also resulted in lower drug accumulation in off-target tissues compared to free drug and larger micelles that were administered either intranasally or intravenously.^[172]

Intranasal administration does have its own limitations, however. A quantitative review of clinical studies conducted over the past 40 years revealed a wide disparity in results, bringing into question the true efficacy and future potential of nose-to-brain drug delivery.^[173] Negative results could be attributed to the hindrance posed by nasal epithelial barriers (including the olfactory surface area, which makes up only 5% of the total nasal surface area in humans),^[13] or to the fast clearance of solutes from the CSF, or to limited drug penetration at neuronal entry points.^[9] Such challenges must be successfully navigated if this strategy of drug delivery is to be fully validated and implemented in clinical practice.

4.2. Crossing the BBB

In the context of drug delivery, there are six main pathways by which molecules can traverse the BBB and enter the CNS, including paracellular transport, passive transcellular diffusion, carrier-mediated transport (CMT), receptor-mediated transcytosis (RMT), adsorptive-mediated transcytosis (AMT), and cell-mediated transport (**Figure 17** and **Figure 18**). Some of these have already been referred to in relation to the transport barrier of the BBB. This section discusses the different pathways in detail and seeks to highlight specific drug

alteration and targeting strategies that can be employed to utilize the various pathways.

4.2.1. Paracellular Transport

As mentioned previously, the paracellular permeability of BMECs under normal physiological conditions is virtually nonexistent.^[22] The intercellular pore size is estimated to be around 1 nm,^[37] suggesting that drug transport between endothelial cells is very limited. However, the permeability of the BBB has been reported to increase under various pathological stimuli, and this may offer a potential opportunity for drug delivery.^[174] Nevertheless, this increase in permeability as part of the pathology of brain disorders is a widely variable phenomenon, and therefore may not be completely reliable for the consistent delivery of therapeutics into the brain.^[40]

4.2.2. Passive Transcellular Diffusion

Passive transcellular diffusion is only available to lipophilic compounds that satisfy very strict criteria, including molecular weights less than 500 Da, cumulative number of hydrogen bonds less than 9–10, and logP values close to 2 (the logP value is the partition coefficient, which measures the level of hydrophobicity or hydrophilicity of a substance).^[4, 175, 176] More specific design parameters will be discussed in the section on drug manipulation, especially with regards to synthesizing lipophilic drug analogues (lipidization).

4.2.3. Carrier-Mediated Transport (CMT)

Normal CMT involves the binding of an endogenous solute to a protein carrier or transporter on the luminal side of the BBB, which triggers a conformational change in the protein and results in the solute being transported to the other side of the membrane. This can occur in the direction of the concentration gradient (i.e., facilitated diffusion transporters), or against the concentration gradient (i.e., active transporters).^[2] A quantitative study of transporter expression levels in the human brain revealed that the most highly expressed solute transporters are EAAT1 and GLUT1.^[52] Another study revealed that hexose and large neutral amino acid carriers have the highest solute capacity.^[177]

These findings lend themselves to drug delivery applications, and suitable design tradeoffs can be established to develop therapeutics that enable maximal brain uptake. However, transporters expressed at the BBB are structure-specific, and as such they rarely transport drug analogues—i.e., drugs that are simply coupled to transporter ligands will usually not be ferried across the BBB, and very few attempts at this have been successful.^[178] To overcome this, drug molecules require modification to mimic the normal ligands whilst still maintaining their innate bioactivity.^[178] One such example is melphalan, a chemotherapeutic drug that has been shown to cross the BBB via the neutral amino acid transporter in tumor-bearing rats.^[179] Small NPs have also been successfully transported across the BBB via CMT, and coating their surface with specific moieties such as compounds derived from choline has proven beneficial for brain uptake.^[178]

4.2.4. Receptor-Mediated Transcytosis (RMT)

RMT is a promising method of drug delivery that involves the endocytotic uptake of macromolecule-sized drugs. Although this approach was initially applied to free drug cargo,^[180] recent attempts have involved the delivery of NPs via the same pathway.^[181] Uptake can occur via clathrin-mediated or caveolin-mediated endocytosis, or alternatively via other pathways including the less explored process of lipid raft internalization (**Figure 19**).^[182-184]

In clathrin-mediated endocytosis a ligand binds to a cell-surface receptor, resulting in the clustering of ligand-receptor complexes in coated pits on the luminal membrane. Clathrin forms a polygonal lattice on the membrane surface, assembled with the help of adaptor protein complexes. After invaginating and pinching off, the vesicle sheds its clathrin coat and forms an early endosome. This endosome then undergoes cell trafficking, and depending upon intracellular conditions can be transcytosed or routed to the lysosomal compartment for degradation.^[185, 186] Although both pathways undergo similar vesicle formation, their

divergent intracellular fates are regulated by specific proteins, including amphiphysin, endophilin, and the various adaptins, dynamins, and rab proteins.^[184, 185] These proteins work collectively to facilitate movement of the endosome along the intracellular cytoskeleton and subsequent fusion with other vesicular compartments along the endolysosomal pathway. The steady progression from early endosome to sorting endosome, to multi-vesicular body, to late endosome and eventually to lysosome is a strictly regulated process marked by specific intracellular environmental conditions. A decrease in pH creates an environment that attracts enzyme function and ultimately results in the degradation of lysosomal contents.^[184] Indeed, clathrin-dependent endocytosis tends to lead to lysosomal trafficking and degradation more so than the clathrin-independent mode of internalization.^[184] However, cationic or pH-sensitive therapeutic agents could possibly prevent routing to the lysosome, thereby ensuring that fusion with the abluminal membrane takes place.^[187]

Caveolin-mediated endocytosis involves the formation of caveolae (small, flask-shaped invaginations in the plasma membrane), which mediate uptake of macromolecules and trigger subsequent intracellular trafficking.^[188] These caveolae contain caveolins, which are integral membrane proteins responsible for regulating the intracellular fate of internalized vesicles^[189]. Caveolae typically follow a defined trafficking pathway to the early endosome, at which point they may be recycled to the luminal surface or further trafficked to other subcellular compartments.^[190] Although the caveolin-dependent pathway was initially suggested to involve the presence of a distinct organelle known as the ‘caveosome’, more recent work suggests that this is not the case.^[189, 191] It is proposed that clathrin-mediated endocytosis is the main pathway of uptake for small molecules with size less than 200 nm, while caveolin-mediated endocytosis is engaged for larger molecules up to 500 nm.^[192] As a result, the caveolin-mediated pathway could offer a useful mechanism for the delivery of

macromolecular drugs (including even uncoated NPs) into the CNS.^[11] The clathrin-mediated pathway, meanwhile, could facilitate entry of smaller, functionalized therapeutic agents.

As mentioned previously, there are several receptors expressed on the BBB that can be exploited for RMT. These include TfR, IR, LRP1, LRP2, LDLR and DT_R, among others.^[193] Some of these receptors, however, are virtually saturated by endogenous ligands under physiological conditions (e.g., IR and TfR), and this makes it difficult for therapeutic agents to bind in adequate amounts.^[194] Nevertheless, RMT can be a feasible strategy if the therapeutic agents to be delivered are functionalized with effective targeting moieties.

4.2.5. Adsorptive-Mediated Transcytosis (AMT)

Since the luminal side of the BBB features a negative charge (due to proteoglycans), electrostatic interactions are triggered whenever a positively charged substance comes into contact with the plasma membrane surface. AMT involves the endocytotic internalization of macromolecules via this pathway, followed by their subsequent passage through the BBB.^[195] Therapeutically, AMT can be achieved in one of two ways: (i) by building cationic surface charge into the drug or NP, or (ii) by conjugating the drug or NP (usually covalently) with a positively charged moiety, such as a cell penetrating peptide (CPP).^[196] CPPs are short targeting vectors that consist of less than 30 amino acids and, despite their overall positive charge, display amphipathic characteristics, thus allowing them to penetrate plasma membranes and transport their cargo into cells.^[197] Both methods to facilitate AMT will be explored in more detail in Sections 5.2.5 and 5.3.3.

Compared to RMT, AMT is a pathway that features lower affinity and therefore lower transcytotic potential (a measure of the drug's ability to traverse the BBB) as a result of weaker interactions with the luminal membrane (although this is highly dependent upon the surface properties of the drug or NP).^[196] However, the vesicles formed during AMT have greater capacity (i.e., they accommodate larger macromolecules) than those formed during

RMT.^[196] Drawbacks associated with AMT include its lack of selectivity (adsorption may occur not only at the BBB, but also in the blood vessels of other organs)^[196] and its potential to increase BBB vascular permeability (due to the possibly toxic effects of positively charged compounds such as CPPs that can be observed when administered in large amounts).^[198]

4.2.6. Cell-Mediated Transport

In the aforementioned pathways, therapeutic agents are strictly designed to minimize entrapment by the immune system, since opsonization often results in drug decomposition and clearance.^[199] Cell-mediated transport is unique in that the objective is the opposite—drug systems are actively engineered to be taken up by immune cells such as monocytes and macrophages.^[11, 199] The rationale behind this is that during common pathological events such as neuroinflammation, leukocytes are extensively recruited and trafficked into the brain parenchyma by processes involving chemotaxis and diapedesis. Since therapeutic agents, especially colloidal carriers, have a tendency to be phagocytosed by these leukocytes, it is proposed that instead of being designed to resist uptake, they could instead be designed to encourage immune cell uptake (**Figure 20**).^[199] The result would be directed targeting to pathologically affected regions of the brain. This “Trojan horse” strategy is capable of delivering both free drug and NPs to the brain, but NP formulations can often be more potent.^[200, 201] Interestingly, this targeting strategy does not require nano-sized carriers to work effectively. Drug systems as large as 1.2 μm can enter the brain,^[200] albeit potentially exhibiting some level of toxicity due to their increased size.^[202] The design specifications for this strategy will be further discussed in the following section.

As an alternative strategy, NPs can be specifically designed to mimic activated leukocytes and therefore penetrate affected regions without needing to be taken up by the immune cells. Various approaches have been examined to achieve this shift towards biomimcry, including formulating drug systems with small subunit cell membrane-derived

molecules (such as peptides and sugars), as well as larger biomacromolecules (e.g., proteins and carbohydrate chains).^[203]

Another promising approach involves using the cell membrane itself as a material for enhancing nanoparticle functionality.^[203] Nonporous silicon NPs, for example, have been shown to be capable of evading the immune system, crossing biological barriers and localizing in target tissues (such as B16 melanoma-affected cells in mice) when coated with leukocyte-derived plasma membranes.^[204] Polymeric NP cores camouflaged with erythrocyte membranes have also been found useful as biomimetic drug delivery vehicles (**Figure 21**).^[205]

Benefits associated with cell-mediated transport include potential for high drug loading capacity; precise, target-specific drug delivery; prolonged drug survival; controlled drug release; and diminished immunogenicity and cytotoxicity.^[199] In contrast, current limitations include degradation and clearance of drugs taken up by immune cells; premature or insubstantial drug release; drug incompatibility with the host immune cell; and off-target drug dispersion (e.g., spleen, liver, blood, lungs), for example in the absence of inflammatory stimulation.^[10, 199] Cell-mediated transport holds significant promise as a novel targeting strategy, but the aforementioned limitations need to be sufficiently addressed before this strategy can become clinically useful in the treatment of neurodegenerative diseases.

5. Strategies for Drug Delivery across the BBB

The six pathways presented in Section 4.2 offer a variety of possibilities to enable or facilitate drug delivery to the CNS by crossing the BBB. In order to deliver most types of drugs to the brain, one of two concessions needs to be made. Either the drug needs to be modified or encapsulated in a suitable carrier to accommodate its passage through the brain, or the brain itself needs to be modified to accommodate the drug. In this regard, three major strategies can be distinguished: (i) BBB manipulation, (ii) drug molecule design and modification, and (iii)

nanomaterial-mediated drug delivery. Despite overlap between the categories, we will endeavor to address them separately in this section.

5.1. BBB Manipulation

This section deals with modifying the BBB physiology to accommodate drug passage.

Manipulation of the BBB can be mainly achieved in two different ways: by opening tight junctions, and by inhibiting the efflux pumps.

5.1.1. Opening of Tight Junctions

The therapeutic strategy of opening tight junctions is based on the twofold reasoning that (i) increased BBB permeability is a phenomenon associated with many neurological diseases, and (ii) as a result, enhanced paracellular transport increases the delivery of small water-soluble molecules into the brain. As discussed previously, the extent to which BBB permeability increases is unpredictable, and often varies from patient to patient and from condition to condition.^[40] However, since the BMECs are much tighter than endothelial cells elsewhere in the body, it is estimated that even under severe pathological conditions, where junctional proteins are downregulated, tight junction permeability only increases to the extent that drug systems less than approximately 20 nm in size can penetrate the BBB.^[11, 206] It is therefore evident that external stimuli are required to facilitate successful paracellular delivery of larger compounds and structures. To that end, there are several different stimuli that can artificially induce the tight junctions to open. These include chemical, biological, and physical stimuli, which are discussed in greater detail in the following paragraphs.

Hyperosmotic Solutions: The intracarotid injection of an inert hyperosmotic solution (such as 25% mannitol or arabinose) artificially creates an osmotic pressure in the brain capillaries. As the vascular environment equilibrates to balance out the ionic concentrations, the endothelial cells shrink and induce widening of the tight junctions, thus increasing BBB permeability.^[14]

When coupled with drug administration (either free drug or NP-based formulations), temporarily enhanced levels of brain penetration are observed. In fact, it has been shown that this approach can result in more than a 20-fold increase in the brain concentration of hydrophilic drugs.^[207] Based on in vivo brain imaging techniques, the therapeutic window during which effective drug delivery can be achieved in humans for this approach is estimated to be approximately 40 minutes, after which time the BBB slowly begins to return to its original level of permeability.^[208] Normal permeability is restored within 8 hours.^[208]

Other Biological and Chemical Stimuli: Several other pharmaceutical compounds can be used to induce transient opening of the BBB. These include biological compounds such as zonula occludens toxin, histamine, bradykinin, Cereport (a synthetic peptide analogue of bradykinin), LipoBridge (a nonimmunogenic formulation containing short-chain oligoglycerolipids)^[40] and VEGF; and chemical compounds such as oleic acid, lysophosphatidic acid and sodium dodecyl sulfate.^[10] These compounds are all delivered via intracarotid injection (except for Cereport, which can also be delivered intravenously^[209]) and many have been observed to exhibit varying levels of time-, dose- and size-dependent efficacy and toxicity^[210, 211].

Focused Ultrasound (FUS): FUS is a noninvasive technique that involves the concentration of acoustic energy at a target region in the body.^[212] Local biological effects can be induced in deep tissues without significant effect to areas outside the field of focus, thus acting as a complement to surgery.^[10, 213] FUS can be produced by thermal and non-thermal mechanisms, and can stimulate local, reversible opening of the BBB when used in conjunction with microbubble contrast agents (**Figure 22**).^[212, 214, 215] These preformed gas bubbles are introduced *prior* to the brain being exposed to FUS, for two reasons: first, the oscillation of microbubbles reflects ultrasound waves when a sonic energy field is applied, thus confining the FUS effect to target regions in the vasculature.^[217] Second, the oscillation and cavitation produced by microbubbles reduces the power needed to open the BBB, making it possible to

apply FUS through the intact skull.^[212, 218] Depending upon the magnitude of the applied acoustic pressure, microbubbles can exhibit either stable cavitation or inertial cavitation (**Figure 23**). Stable cavitation occurs at low pressures, and results in transient opening of the BBB without causing vascular or neuronal damage. Conversely, inertial cavitation occurs at high pressures, and tends to result in violent microbubble oscillation leading to sudden collapse and potential damage to the surrounding microenvironment.^[219] In vivo mouse model studies have demonstrated that the BBB can be disrupted safely and transiently when acoustic pressures under 0.45 MPa are applied in conjunction with microbubbles measuring no more than 8 μm in diameter.^[220, 221] Other studies have indicated that optimal transcranial focusing can be achieved at frequencies less than 1 MHz.^[7, 218] This technology can be combined with magnetic targeting techniques to effectively target drug systems into the brain parenchyma. For example, it was found that ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles, when incorporated into microbubbles, could magnetically guide drug systems to target regions of the murine cerebral vasculature, after which time the transcranial FUS pulse could generate localized opening of the BBB.^[13, 222]

Microwave Field: Microwave irradiation is another strategy that presents the ability to transiently open the BBB. In animal studies, Moriyama et al. discovered that microwave-induced hyperthermia can produce temporary opening of the BBB at temperatures above 40.3 °C.^[223] Other studies also suggest that the brain needs to be made hyperthermic in order for changes in permeability to occur.^[224] Although microwave-induced BBB opening is a potentially feasible drug delivery strategy, exposure of the brain to thermal microwaves has significant safety implications that need to be addressed in further studies.

Electromagnetic Field (EMF): EMF waves have been shown to transiently induce opening of the BBB,^[225] with both increased frequency and increased amplitude modulation of the input wave corresponding to enhanced BBB permeability.^[226] In addition, pulse waves have been

shown to be more effective at eliciting a response than continuous waves.^[226] In light of these initial studies, the underlying pathophysiological implications of EMF-induced drug delivery must be further explored to ensure its suitability for clinical application.

Despite these varied options that may feasibly permit paracellular drug transport, manipulation of the BBB through opening of the tight junctions is a rather non-specific and non-selective strategy. Although BBB opening has the potential to allow therapeutic agents to be delivered into the brain in efficacious amounts, it can also enable unregulated influx of other compounds, including pathogens and neuroinflammatory mediators.^[227] As a result, CNS-linked pathologies may be exacerbated rather than ameliorated. For example, the administration of a hyperosmotic solution in cancer patients was observed to cause seizures in 7% of cases where patients were originally seizure-free.^[14, 228] Another issue is that while the techniques listed above may increase the paracellular permeability of BMECs, few—if any—actually act on the efflux transporters at luminal and abluminal membranes. These transporters, especially P-gp, remain active at normal or elevated levels (e.g., due to pathology), and may in fact compensate for the increased paracellular trafficking.^[28] As such, therapeutic agents that serve as substrates of P-gp, even if successfully delivered in combination with BBB manipulating stimuli, may still be effluxed from the brain.^[229]

5.1.2. Inhibition of Efflux Pumps

In humans, the primary efflux transporters that are present at the BBB include BCRP, P-gp and the other MRPs, in order of their typical expression levels.^[52] Collectively, these efflux systems work to keep a whole host of drugs from successfully reaching the brain parenchyma. P-gp serves as a transporter for several lipophilic molecules, whilst many MRPs are responsible for transporting both charged and neutral compounds, but especially anionic compounds.^[21] Many drug candidates that might otherwise achieve entry into the brain are hindered by these transporters, and although increased BBB permeability may somewhat

increase their entry, alternative approaches have been sought to overcome the challenges posed by the efflux systems.^[229] One such approach is inhibition of the efflux pumps (**Figure 24**).

Both endogenous and exogenous stimuli have been found capable of inhibiting primary efflux pumps and, when administered in conjunction with otherwise unsuccessful drug candidates, achieve increased brain concentrations of those drugs. Endothelin-1 (ET-1), for example, is an endogenous receptor antagonist that downregulates P-gp activity at the BBB.^[28] When delivered in combination with a therapeutic agent, recombinant ET-1 could potentially serve as a successful drug delivery mechanism. However, the pathological implications associated with increasing levels of this endogenous compound in vivo must be further considered before clinical application becomes feasible. For example, in addition to acting at the BBB, ET-1 also acts systemically, such as in pulmonary vessels to cause vasoconstriction.^[231] Thus, increasing ET-1 levels to improve drug delivery to the brain could simultaneously cause off-target effects in other parts of the body. Other examples of efflux inhibitors include broadly-acting first-generation compounds such as verapamil, quinine and quinidine,^[232, 233] as well as more pharmacologically specific second- and third-generation compounds such as PSC-833 (valsopodar),^[234] GF120918 (elacridar),^[235] and XR9576 (tariquidar).^[236] Similar risks, including inhibitor-mediated toxicity, over-accumulation of compounds and off-target effects, have been associated with the use of these small molecule inhibitors.^[237] Nevertheless, a comprehensive review of preclinical and clinical CNS drug interaction studies conducted in 2013 concluded that adverse CNS interactions are generally unlikely to occur as a result of efflux transporter inhibition.^[238]

Alternative strategies to inhibit efflux transporters include the co-delivery of siRNA via poly(glutamic acid) (PGA) and mesoporous silica NPs,^[239] as well as administration of exogenous poloxamers (block copolymers, also known by the trade names ‘Pluronics’,

‘Synperonics’ and ‘Kolliphor’). Poloxamers are versatile and widely-used industrial agents that comprise alternating ‘blocks’ of hydrophobic propylene oxide (PO) and hydrophilic ethylene oxide (EO), arranged in a tri-block formation (EO-PO-EO) (**Figure 25A**).^[240] The PO:EO ratio determines the hydrophobic-hydrophilic balance and ultimately gives the copolymers their amphiphilic nature.^[241] This balance can be changed by modifying the lengths of the PO and EO segments, which in turn changes the ability of the copolymers to interact with the surrounding biological environment.^[241] A salient feature of poloxamers is their ability to exist in two forms: (i) solution form, as individual block copolymers (unimers), and (ii) aggregate form (e.g. as micelles). They are found in solution at low temperatures and concentrations (below the critical micelle temperature and critical micelle concentration), whereas they self-assemble into micelles (either spherical or wormlike, depending on the composition) after reaching the critical micelle temperature and concentration.^[241] Poloxamers can function as conjugated drug delivery vehicles when in micelle form (Figure 25B), while they are typically co-administered with therapeutic agents when in unimer form.^[242] They have been shown capable of inhibiting P-gp, as well as MRP1 and MRP2 albeit to a lesser extent.^[243-247] It is believed that they are most effective at preventing efflux when taken up in unimer form.^[242]

Co-administration of therapeutic agents (that are substrates of these efflux systems) with poloxamers can result in their temporarily but significantly increased uptake into the brain.^[245] The mechanism of inhibition is believed to involve copolymer interaction with the cell membrane, followed by inhibition of P-gp ATPase activity and depletion of cellular ATP.^[243] It has been observed that lipophilic poloxamer with PO length between 30–60 units and hydrophobic-hydrophilic balance less than 20 (PO:EO < 20) serves as the most effective inhibitor of P-gp activity.^[248] Although the clinical viability of poloxamers in the context of brain diseases remains under investigation, their promise is evident. In phase I and II clinical

studies, poloxamers in micellar form have been successfully used to deliver the anticancer drug doxorubicin in patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction, and the resulting lack of toxicity has been well-documented.^[249, 250] The inhibitory effects of poloxamers have been shown to be transient, only affecting P-gp activity and ATP levels for a short period of time, and without any observable compromise to BBB integrity.^[244]

Yet despite the benefits associated with exogenous efflux inhibitors, there remain some lingering questions about the potential downsides of the technology. For one, if chronically administered, poloxamers could cause prolonged inhibition of P-gp and other efflux pumps. This could lead to the unregulated inflow of other, unwanted substances, not only therapeutic agents, thus causing interference with physiological homeostatic mechanisms and ultimately leading to neurotoxicity.^[251] In light of this, it has been suggested that therapeutic strategies involving efflux inhibition are best suited to the treatment of acute diseases, such as brain tumors, where the aim is to maximize brain drug concentrations for a short period of time.^[251]

5.2. Drug Molecule Design and Modification

This section will consider some of the modifications that can be made to drug molecules to facilitate their uptake into the brain. Understandably, many targeting strategies that can be applied to free drug cargos can also be applied to NPs, and therefore some of the strategies explored in this section may overlap with those in the next section (including CMT, RMT and AMT strategies). In the interests of succinctness and brevity, and to prevent unnecessary repetition, the strategies that incur overlap will only be discussed in detail once, in the section wherein they are more readily applied.

5.2.1. Drug Lipidization

Lipidization is the process by which a drug is modified such that it can undergo passive transcellular diffusion. It is a complex process, one that requires an equilibrium to be struck to achieve sufficient, yet not excessive, lipophilicity. Insufficiently lipid-soluble drugs fail to penetrate the plasma membranes of the BBB, whilst excessively lipid-soluble drugs become sequestered in peripheral and BMEC membranes. To ensure maximal brain penetration it is estimated that the ideal ratio for brain extraction, measured by the octanol/water coefficient, should be somewhere between 10:1 and 100:1.^[28] There are many distinct parameters that define a successful lipophilic analogue (**Table 1**), according to Pajouhesh and Lenz,^[175] taking into account Lipinski's "Rule of 5". Various techniques have been utilized to enhance the lipid solubility of prospective drug candidates (**Table 2**).^[177]

Table 1. A summary of the key parameters that define a successful lipophilic analogue

Parameters	Stipulations	References
<i>Molecular weight (MW)</i>	Exact stipulations vary within the literature, but MW should generally be kept in/below a range of 400–600 Da (average MW for marketed CNS drugs is 300–400 Da).	[175, 252, 253]
<i>Lipophilicity</i>	The logP value (the oil/water partition coefficient, which measures the level of hydrophobicity/hydrophilicity of neutral molecules) should be between 1.5–2.7 to facilitate optimal brain uptake. The logD value (the oil/water distribution coefficient at physiological pH) should be between 0–3.	[175, 254]
<i>Hydrogen bonding</i>	Increased H-bonding decreases lipophilicity, and hence BBB penetration. CNS penetration therefore requires a total of	[175, 176]

	less than 5 heteroatoms (atoms that are not H or C). Clinical drug studies suggest that less than 3 H-bond donors (sum of OHs + NHs) and less than 7 H-bond acceptors (sum of Ns + Os) should be present.	
<i>Polar surface area (PSA)</i>	CNS drugs tend to have a lower PSA than other drug classes. The PSA required for successful BBB penetration is around 60–90 Å ² . Evidence suggests that BBB permeability decreases 100-fold as the PSA is increased from 52 Å ² to 105 Å ² .	[146, 175, 254, 255]
<i>Molecular volume and flexibility</i>	These properties respectively define the conformational structure of molecules under physiological conditions, and the ease by which molecules travel through lipid bilayers. Most CNS drugs have less than 5 rotatable bonds, but the upper limit is 8. Limited, but not overt, flexibility is advantageous to drug delivery.	[175, 253, 256]
<i>Charge</i>	Since CSF is slightly more acidic than plasma, the uptake of bases is somewhat favored. A positive charge at pH between 7–8 is ideal for drug candidates. pKa should be between 7.5–10.5.	[28, 175, 257]
<i>Metabolic stability</i>	This refers to a drug's ability to withstand first-pass hepatic metabolism (both phase I and phase II) when administered via the enteral route. The ideal candidate maintains over 80% of its initial concentration after 60 minutes.	[175, 258]
<i>Metabolic</i>	The superfamily of CYP enzyme isoforms is heavily	[175]

<i>liability</i>	involved in phase I hepatic metabolism. For optimal oral absorption, a drug candidate should not elicit significant CYP2D6 metabolism and should not be a potent CYP3A4 inducer. Drugs that inhibit the metabolic activity of CYP enzymes should exhibit less than 50% inhibition at a concentration of 30 μM .	
<i>Protein binding</i>	Protein binding is an important consideration as drugs move through the vasculature. Ideal CNS drugs should not be efficient P-gp substrates, and should not be high affinity ($K_d < 10 \mu\text{M}$) serum albumin ligands.	[175, 259]
<i>hERG inhibition</i>	Drugs that interact with cellular ion channels can potentially block hERG, a gene that codes for the K^+ ion channels. These K^+ channels are necessary for the proper functioning of the cardiac cycle and, when upset, can result in cardiac arrhythmias. When designing drug candidates, there should be more than a 30-fold buffer between the hERG IC_{50} and effective unbound plasma concentrations.	[175, 260]
<i>Aqueous solubility</i>	Drug solubility should be greater than $60 \mu\text{g mL}^{-1}$ to ensure maximal brain uptake.	[175]
<i>Shape</i>	The optimal drug candidate should take on a length/width ratio of less than 5.	[254]

Table 2. A summary of various strategies for enhancing the lipid solubility of prospective drug candidates

Strategies	Rationale	References
<i>Cyclization</i>	Cyclization of peptides may reduce their H-bonding, increase their lipophilicity, and reduce their hydrodynamic radius in solution, thus enhancing their passage through the BBB.	[177, 261]
<i>Halogenation</i>	Halogenation of peptides such as biphalin has shown significantly increased brain uptake in animal experimental models, in a manner that was proved to be linked to the conjugated halogen.	[177, 262]
<i>Acylation</i>	Acylation of the N-terminal of peptide drugs can increase their BBB permeability.	[177, 263]
<i>Methylation</i>	Methylation can irreversibly block hydroxyl groups on drug molecules, thereby increasing their lipid solubility. Methylation of thyrotropin releasing hormone (TRH) analogue was found to increase plasma and brain stability and enhance activity in the CNS.	[177, 264]
<i>Increased alkyl chain length</i>	The addition of non-polar groups at sites that do not interfere with receptor-binding regions can result in increased lipid solubility. For example, increasing the length of the aliphatic chain of <i>n</i> -alcohols from 1 to 8 carbons results in a fourfold increase in lipid solubility. However, the tradeoff is that a drug may become too large, thereby	[177, 265]

	reducing passage across the BBB.	
<i>Amino acid addition or substitution</i>	The overall balance of polar to non-polar groups within a drug molecule can be reduced by substituting less charged amino acids for charged amino acids, or alternatively by adding uncharged amino acids to an existing peptide.	[177, 266, 267]
<i>Fatty acid or cholesterol ester</i>	In contrast to methylation, esterification can reversibly block hydroxyl groups on drug molecules, rendering them more lipid-soluble.	[177, 268]

Despite the historic popularity of lipidization in the context of drug delivery, there are some challenges that remain. Foremost is the issue of efflux mechanisms. Many of the strategies listed above can successfully increase lipid solubility of drug candidates, but this often comes at the expense of making them better substrates for the P-gp efflux system.^[28] Ways to overcome this include co-administration with efflux inhibitors, as well as exploration of other pathways across the BBB (e.g., CMT, RMT, AMT). A second issue is that lipid bilayers are of course not only found in BMECs, but also in all other cells in the body. Thus, oral or systemic administration of a lipophilic drug analogue is likely to be non-specific and therefore result in unwarranted drug sequestration in peripheral tissues.^[269, 270] Intra-arterial administration could potentially lessen these effects, since its location closer to the head may prevent excessive drug dispersion to peripheral regions.

5.2.2. Prodrugs

Prodrugs are chemical compounds that form pharmacologically active drugs only after being metabolized. They are synthesized to overcome a range of different issues, including low bioavailability due to poor absorption from the gastrointestinal tract,^[271] degradation by protective mechanisms after reaching the targeted site,^[177] and widespread systemic exposure

resulting in significant off-target effects.^[272] Prodrugs are formulated by reversibly attaching a distinct moiety to a drug compound, which can subsequently be removed via enzymatic cleavage or hydrolysis to allow the drug to induce its effect (**Figure 26A**). Transport across the BBB can occur via any one of the six pathways (paracellular transport, passive diffusion, CMT, RMT, AMT or cell-mediated transport), depending upon the makeup and functionality of the original drug compound.^[14] Prodrug design for brain drug delivery can be approached in different ways (Figure 26B). Esterification (as well as amidation) of amino, hydroxyl, or carboxylic acid-containing drugs can enhance lipid solubility, and has shown promise in avoiding the abundance of endogenous esterases in the CNS.^[177] Once across the BBB, these prodrugs can then be hydrolyzed to release their active compounds. Aromatic benzoyl and tert-butyl esters have shown stability in the plasma, while inducing cleavage and thus activation within the CNS. Lipophilic amino acids, such as phenylalanine, can also be added to drug molecules as the cleavable unit, resulting in increased drug diffusion through the BBB.^[177]

An alternative strategy is to utilize the redox system of the brain. Lipophilic molecules, including methyldihydropyridine, can be conjugated to drug compounds, resulting in their increased uptake through the BBB. After reaching the brain, these prodrugs are then oxidized into a hydrophilic quaternary form that effectively “locks” them into the CNS compartment and prevents them from escaping back into the vasculature.^[177]

Recent studies have proven the potential viability of prodrugs in the context of GBM. Brain tumor cell growth depends upon glutamine levels, since glutamine is a key contributor to the tricarboxylic acid (TCA) cycle and to the various biosynthetic pathways (including synthesis of nucleotides, proteins and lipids).^[272] Thus, Rais et al. postulated that inhibition of glutamine utilization should also inhibit tumor cell growth in vitro and in vivo.^[272] For this purpose, 6-diazo-5-oxo-l-norleucine (DON) was formulated. DON is a non-natural amino

acid, structurally similar to glutamine and capable of alkylating several glutamine-using enzymes including glutaminase, NAD synthase and CTP synthase. Its administration has been shown to robustly inhibit glutamine-dependent cancer cell growth (> 50% tumor reduction) in patients with late-stage GBM,^[274] but this comes at a significant cost. Dose-limiting toxicity is a side effect of DON administration, primarily impacting the glutamine-dependent gastrointestinal tract.^[272] Symptoms include weight loss, hunching, ptosis and lethargy.^[272] Thus, a selective drug delivery system is required; one that maximizes brain exposure yet minimizes systemic exposure. To achieve this, Rais et al. formulated a family of DON prodrugs, each member of which comprised different prodrug moieties. After analyzing the pharmacokinetic profiles of each, it was found that the most effective DON prodrug consisted of two moieties (rather than one). Pivaloyl-oxyl-methyl (POM) esterification of the carboxylate and amine groups on the original molecule resulted in a stable compound that retained its ability to convert back to DON.^[272] Evaluated in monkeys, this prodrug achieved a 7-fold lower plasma exposure but 10-fold higher CSF exposure when compared to DON.^[272]

Two of the challenges associated with this targeting strategy include the peripheral sequestration of lipophilic prodrugs, and the potential instability of synthesized prodrug compounds. If these can be overcome, e.g. by precise choice and placement of cleavable moieties, prodrugs offer a promising approach to CNS drug delivery.^[275]

5.2.3. Analogue-Based Drug Design for CMT

The carrier or transporter mediated system of transport across the BBB has already been discussed to some extent in Section 4.2.3. Many facilitated diffusion transporters and active transporters are present in varying proportions at the endothelial cells, and they manage the selective uptake of nutrient molecules into the brain parenchyma. As such, they can serve as portals of entry for smaller drug systems. The restrictive nature of BBB transporters means that drug systems are required to mimic the molecular structure of endogenous nutrients.^[178]

For this reason, CMT strategies are better suited to free peptide drugs, which are more easily able to conform to the structure of nutrient molecules.^[177] Levodopa is perhaps the most well-known example of a free drug with a CMT targeting strategy. Being a lipid-soluble precursor of dopamine, it contains carboxyl and α -amino groups that enable it to compete for transport across the BBB by the large neutral amino acid transporter.^[177] Many such transporters can be targeted for blood–brain transport. GLUT1 constitutes over 90% of BBB glucose transporters.^[146] EAAT1 is also highly expressed at the BBB.^[52] Hexose and large neutral amino acid carriers have the highest solute capacity.^[177] LAT1 and CAT1 are the principle large neutral and cationic transporters.^[146]

Studies that quantitatively measure transporter expression levels at the human BBB should be taken into account during drug design, since high expression levels combined with high solute capacities are most conducive to effective CNS transport. In addition to free drugs, NPs of smaller size could also be functionalized to exploit this method of crossing the BBB.^[177] However, CMT drug targeting is a generally less favored therapeutic approach for a few reasons. Firstly, transporters impose significant restrictions on drug size and shape, permitting transport only of small molecules that take on the conformation of their endogenous substrates.^[178] Secondly, transporters accommodate much lower drug quantities than vesicles formed during the course of RMT (Section 5.2.4) and AMT (Section 5.2.5), thus necessitating more frequent drug administration.^[177] Thirdly, successful drug delivery via CMT may interfere with endogenous nutrient transport, potentially resulting in CNS complications as a result of disturbed parenchymal nutrient levels.^[10, 276]

5.2.4. Functionalization with Receptor-Targeting Ligands for RMT

RMT targeting strategies were originally only applied to free drugs,^[180] but they have now been extended to NP-based drug systems with comparable levels of success.^[181] As such, most of the RMT drug targeting strategies will be discussed in the context of NP

functionalization (Section 5.3). Note that similar brain-targeting ligands that are used to functionalize NPs can also be conjugated to free drug molecules to enable receptor-mediated uptake, and vice versa.

However, one approach that will be briefly highlighted here is antibody targeting. Monoclonal antibodies (mAbs) are antibodies produced by immune cells that are all clones of a unique parent cell. They have monovalent affinity, in that they bind to a single type of epitope (the specific segment of protein that forms the antibody binding target on antigens and other proteins). In the context of BBB transport, mAbs can bind very specific exofacial epitopes that are located on different luminal receptors. This receptor-specific binding may enable the mAbs, along with everything conjugated to them, to piggyback across the BBB via the RMT system.^[146] Compared to endogenous ligands such as insulin and transferrin, mAbs may, if directed against suitable antigens, constitute safer and more effective targeting vectors since they do not have to compete with other ligands for the receptor-binding sites.^[9, 277] The exofacial epitopes are spatially removed from the binding sites, ensuring that mAbs have direct and unhindered access to the receptors whilst still permitting endogenous ligands to bind.^[146]

Among others, one strategy for attaching two proteins together, each of which has a different functionality (i.e., targeting functionality and therapeutic functionality), involves using recombinant fusion protein technology. Several free peptide drugs have been conjugated with targeting mAbs in an effort to safely increase their uptake into the brain, including vasoactive intestinal peptide (VIP), brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), β -galactosidase, and many others.^[278] It is believed that the choice of mAb is critical to the success of the strategy, since some mAbs (e.g., those targeting LRP1) only trigger endocytosis, whereas others trigger full transcytosis from luminal to abluminal membrane.^[54] To date, the most successful mAbs

appear to be those directed against TfR, human IR (HIR), and the FC5 orphan receptor (the BBB receptor for the FC5 antibody has not yet been identified, but is believed to be the cell cycle control 50A protein, also known as the transmembrane protein 30A (TMEM30A)).^[54] A study by Yu et al. suggests that at therapeutic drug concentrations, the amount of brain uptake is inversely dependent upon the affinity of the antibody for its receptor.^[279] Using a murine model, Yu et al. administered therapeutic doses of anti-TfR mAbs and found that high affinity mAbs remained closely associated with the BBB whereas lower affinity variants were trafficked into the CNS to a much greater extent.^[279] These lower affinity mAbs showed an almost fivefold increase in brain drug concentration compared to the high affinity mAbs, thereby indicating the importance of precisely tuning antibody affinity to ensure robust CNS uptake.

Historically, one of the problems associated with mAbs has been their large size, which can potentially cause conjugated macromolecules to diffuse poorly through biological membranes.^[280, 281] (Inherent immunogenicity also previously posed a challenge to therapeutic development, however, recent advances—including the development of fully humanized antibodies—have rendered this less of a problem nowadays.^[282-284]) Single-domain antibodies (sdAbs) have been formulated in an attempt to address the issue of sizing constraints. sdAbs function similarly to normal antibodies, but only contain a single monomeric variable antibody domain. This structural property renders them lightweight (12-15 kDa), around ten times lighter in molecular weight compared to regular IgG antibodies (150-160 kDa).^[285] Despite their smaller makeup, sdAbs have proven capable of having similar specificity as normal antibodies, and in some cases have demonstrated greater robustness.^[286] A prime example is the FC5 sdAb, which transmigrates across human BMECs in a polarized, charge- and temperature-independent manner, thus suggesting RMT.^[278] The low molecular mass of sdAbs is also believed to confer on them better permeability in tissues, greater stability,

considerable heat-resistance, and lower immunogenicity than whole antibodies. They also do not show complement system triggered toxicity, since they lack an Fc region.^[287]

Although currently no FDA-approved recombinant biologic that can act *behind* the BBB exists,^[54] these aforementioned properties make drug targeting using antibodies and antibody-like affinity proteins (including designed ankyrin repeat proteins (DARPs)) a promising strategy for future therapeutic discovery and development.^[288-291]

5.2.5. Functionalization with Cationic/Amphiphilic Constituents for AMT

Similar to RMT, AMT-based strategies can be applied to both free drug molecules and NPs. Cationization and conjugation of CPPs, the two principle methods by which AMT is achieved, can be used to transport small molecules, proteins, peptides, fragments of DNA and NPs across the BBB.^[196] Since these strategies are increasingly being applied in the context of NP functionalization, they will be explored more fully in the next section.

One strategy that will be discussed here, however, is glycosylation. Primarily used with free peptide drug molecules, glycosylation has proven to be a useful methodology for enhancing BBB permeation and increasing biodistribution to the brain.^[292] It involves the conjugation of carbohydrate chains to core peptide molecules, resulting in a compound that is less lipophilic (and thus less capable of undergoing passive diffusion) but that is capable of achieving BBB transport via another pathway.^[177] The exact mechanism by which this transport occurs remains to be elucidated, but the amphiphilic nature of glycopeptides seems to lend itself to AMT.^[177, 292] It has been confirmed that passive diffusion and CMT are *not* involved in transporting glycopeptides across the BBB.^[177] Although different carbohydrate chains (including glucose and xylose) produce inherently different distribution patterns, they generally improve the water solubility, stability and bioavailability of peptide analogues. Effective glycopeptides contain an amphipathic state that promotes adsorption to biological membranes, as well as a random coil state that is water-soluble.^[292] It has been shown that a

glycopeptide derivative of endomorphin-1 (an endogenous opioid peptide), synthesized by N-terminal attachment of lactose succinamic acid, is capable of producing a 700-fold increase in BBB permeability and 21-fold increase in plasma stability compared to the native peptide when administered via the oral route.^[292]

5.3. Nanomaterial-Mediated Drug Delivery

Although countless free drugs (e.g., peptides, proteins, genes, antisense drugs) have been synthesized in an effort to combat CNS disease, many of those drugs have been rendered ineffective by their unfavorable in vivo properties. Poor stability in biological fluids, rapid enzymatic degradation, inadequate release profiles and unfavorable pharmacokinetic properties are among the many reasons that such drugs may fail to achieve clinical efficacy.^[15] In order to overcome these challenges, nano-sized carriers are increasingly being developed to protect and target drug molecules that are ineffective on their own. Drugs may associate with NPs by being adsorbed, dissolved, encapsulated or bound covalently.^[192] For example, in the context of layer-by-layer (LbL) capsule assembly, drug loading can be separated into three broad categories: (a) integration of cargo with the capsule wall, (b) preloading of the capsule template with cargo prior to LbL assembly, and (c) postloading of the capsule by altering its permeability and trapping cargo inside (**Figure 27**).^[293] There are several different types of NPs, each of which boasts unique pharmacodynamic characteristics. Yet despite their differences, most (if not all) NPs that act behind the BBB and exhibit some degree of efficacy in vivo adhere to a general set of guidelines that are summarized in **Table 3**. Many of these properties are not inherently present in the NPs themselves, and therefore specific moieties that endow these added functionalities can be conjugated to their surface or incorporated within their nanostructures (e.g., by avidin-biotin binding, heterobifunctional linkers, or “click” chemistries).^[280, 294-296] The integration of various functionalities into the

one drug system is known as NP functionalization, and this approach has proven advantageous in enhancing drug delivery to the brain.

Table 3. A summary of the general guidelines for designing NPs that can deliver drugs across the BBB

Guidelines	References
NPs should be <i>non-toxic</i> and <i>biocompatible</i> . Biodegradability, although often a desirable property, is not always essential, since for example inorganic NPs can sometimes be renally excreted instead of degraded.	[13, 15]
Although NPs can range in size from 10–1000 nm, the <i>diameter should generally be less than 100 nm</i> (except if engaging in cell-mediated transport, in which case the vesicle size can be up to ~1 μm). At sizes greater than 100 nm, passage into the brain extracellular space may be restricted due to the narrow width of this space (~40–60 nm in the healthy brain). Conversely, NPs that are too small (< 5 nm) can potentially enter peripheral cells unchecked, or be rapidly removed from the body via renal clearance. Certain studies (conducted with various types of NPs) suggest that <i>optimal cell association and endocytotic uptake occur for NPs ~50 nm in size</i> . Nevertheless, the exact NP sizing criterion for optimal BBB transport is still somewhat disputed.	[15, 40, 188, 200, 280, 297-302]
NPs should maintain <i>good stability in blood</i> (limited aggregation or dissociation).	[13, 15, 303]
NPs should <i>avoid being taken up by immune cells</i> (unless interacting with the purpose of stimulating anti-pathogenic immunity or engaging in cell-mediated transport) and, if administered via intravenous or intracarotid	[13, 304, 305]

injection, should generally possess <i>long blood-circulation times</i> .	
NPs should be <i>able to deliver functional cargo (e.g., small molecules, peptides, proteins and/or nucleotides)</i> .	[13, 15, 303]
NPs should achieve <i>targeted delivery of drugs across the BBB</i> .	[15, 303]
NPs should cause <i>minimal or reversible drug alteration (e.g., chemical degradation, protein denaturation)</i> .	[13, 15, 303]
NPs should possess <i>tunable or application-specific drug release profiles</i> .	[13, 15, 303]
NP <i>manufacturing should be a scalable and cost-effective process</i> .	[13, 15, 306]

5.3.1. Types of NPs

This section, although it is not intended to be exhaustive, will explore and provide an overview of different types of NPs (**Figure 28**), including lipid-based NPs (Figure 28A), polymeric NPs (Figure 28B), and inorganic NPs (Figure 28C). The therapeutic implications of using these NPs to cross the BBB in brain drug delivery applications will be further explored in Section 5.3.2 and Section 5.3.3.

Lipid-Based NPs: Lipid-based NPs are typically stable, non-toxic carriers that are well suited to brain drug delivery applications. Among these, the most common types are liposomes and solid lipid NPs (SLNs), both of which are discussed below.

Liposomes

Liposomes are spherical vesicles that consist of one or more lipid bilayers (known as lamellae) bounding an internal aqueous space. They are commonly composed of amphiphilic phospholipids such as sphingomyelin and phosphatidylcholine.^[307] Cholesterol is also frequently included in liposomal formulations, as it has proven to increase stability in vivo.^[311] Liposomes are generally subcategorized on the basis of their size and number of lamellae. Small unilamellar vesicles (SUVs) have sizes up to 100 nm and one lipid bilayer,

large unilamellar vesicles (LUVs) are larger than 100 nm and contain one bilayer, and multilamellar vesicles (MLVs) are often over 500 nm in diameter and contain several concentric bilayers (**Figure 29**).^[312, 313]

Depending upon the lipids used in formulation, liposomes can be neutral, anionic or cationic. For cationic liposomes, one of the most commonly used lipids is 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), mixed with dioleoyl-phosphatidylethanolamine (DOPE).^[315] Indeed, the positive charge associated with cationic liposomes enables them to interact more effectively with the negatively charged surface of BMECs. This renders them capable of accumulating in the brain in greater amounts, but also presents potential issues including decreased stability, increased cytotoxicity in vivo (even though liposomes generally possess low toxicity), and increased non-specific cell binding.^[316] Without being functionalized, many liposomes are rapidly cleared from circulation by the reticuloendothelial system (RES), sometimes also referred to as the mononuclear phagocyte system (MPS) (containing phagocytic immune cells).^[316, 317] One study found that negatively charged liposomes were more rapidly sequestered by circulating monocytes than both positively charged liposomes (3-fold faster) and neutral liposomes (5-fold faster).^[318] This tendency towards increased association of negatively charged liposomes with monocytes has been corroborated by other studies.^[319-321] Decreasing liposomal size (<100 nm) and surface functionalizing with specific moieties have both proven effective at extending circulation times and improving brain drug targeting.^[316] In studies conducted by Gao et al., doxorubicin-loaded liposomes measuring ~180 nm in size were conjugated with two targeting ligands, transferrin and folate, before being administered intravenously in tumor-bearing rats.^[322] Since TfRs are reportedly overexpressed at the luminal side of the BMECs,^[323] transferrin was selected with the purpose of facilitating efficient RMT across the BBB. Liposomes were further modified with folate to promote uptake by tumor cells, since the folate receptor (FR)

has been shown to be overexpressed in a variety of human tumors.^[324, 325] Results showed that treatment with dual-functionalized liposomes enabled more effective delivery of anti-cancer agents to the brain, evidenced by the longer median survival time of rats (30 days) compared to those treated with saline solution (20 days), doxorubicin solution (24 days), and uncoated doxorubicin-loaded liposomes (27 days).^[322] Additionally, magnetic resonance imaging (MRI) assessment showed that the dual-functionalized liposomes exerted significant tumor suppressive effects on glioblastoma cells, resulting in the reduced size of tumor regions.^[322] Toxicity studies confirmed that the liposomal formulation did not induce any observable toxic effects in systemic organs, not even in the heart or liver;^[322] this was an important finding, because doxorubicin has elsewhere been reported to cause cardiotoxicity when administered in free drug form.^[326] In separate studies, the brain targeting efficacy of five BBB receptor-targeting ligands (transferrin, RI7217, COG133, angiopep-2, and cross-reacting material 197 (CRM197)) was investigated in vitro and in vivo after conjugation with liposomes.^[327] Each liposome was decorated with an estimated number between 22 and 25 ligand molecules. Results indicated that of the 5 ligands, only RI7217, which is an anti-TfR antibody, achieved significant and prolonged binding to human BMECs in vitro and accumulation in the mouse brain in vivo.^[327] For these RI7217-coated liposomes, BMEC uptake was up to 10 times higher than uncoated liposomes, and parenchymal uptake was up to 4.5 times higher (at 12 hours post-injection, the percentage of initial dose that accumulated in the brain was 0.18% for RI7217-coated liposomes, and 0.04% for untargeted liposomes).^[327] Although the liver was the primary site of off-target organ distribution at all time points, there were no major differences in systemic biodistribution profiles between coated and uncoated liposomes. In consideration of these results, there are a number of variables that may influence brain targeting efficacy in vivo. These include the ligand density introduced in formulation; the unique distribution pattern of receptors at the BBB, which varies between species; and the

type of NP used, due to heterogeneous size, shape and surface properties.^[327] Indeed, certain drawbacks associated with liposomes include the significant number of excipients and complex preparation procedures sometimes associated with their formulation, their relatively low physical stability, and challenges associated with controlling and sustaining drug release.^[15, 312]

Solid Lipid Nanoparticles (SLNs)

SLNs are spherical, stable nanocarriers that possess a solid hydrophobic lipid core matrix stabilized by aqueous surfactants.^[328, 329] The core is typically composed of biocompatible lipids such as triglycerides, fatty acids and waxes, which have the ability to solubilize lipophilic molecules. The stabilizing surfactants, on the other hand, are composed of biological membrane lipids such as phospholipids, sphingomyelins, bile salts and cholesterol.^[330] Drugs can be dissolved or dispersed into SLNs. Benefits associated with this type of NP include its biocompatibility, significant drug entrapment efficiency (higher than many other NPs), increased drug stability, and the ability to provide controlled drug release over a timescale of several weeks (due to the increased mass transfer resistance offered by the solid state of the lipid).^[331] As with many other NPs, SLNs can be modified by surface functionalization to limit RES uptake and improve specific targeting to the brain. In vivo studies using rats conducted by Jose et al. showed that SLNs, after surface modification, were able to significantly increase the distribution of resveratrol within the brain.^[332] The optimal formulation was deemed to be the resveratrol-loaded SLN with drug:lipid ratio of 1:10 and particle size less than 250 nm, and functionalized with a combination of two surfactants—polysorbate 80 (PS 80) and poly(vinyl alcohol) (PVA).^[332] This drug formulation demonstrated a relatively high encapsulation efficiency (~30%, measured as the percentage of drug incorporated into SLNs relative to the total drug added) and loading capacity (~3%, measured as the percentage of drug incorporated relative to the SLN weight), a similar

cytotoxicity profile to free resveratrol (the SLN itself exerted no cytotoxic effects), low accumulation in systemic organs (potentially attributable to PS 80's hydrophilicity that may have contributed to a reduction in NP uptake by RES organs), and most importantly, significantly increased brain drug accumulation compared to free drug (6 times higher accumulation of resveratrol in parenchymal tissue).^[332] Surface charge also appears to play a role in determining the extent to which both SLN-mediated brain uptake and toxicity occur. In murine studies, anionic and cationic tripalmitin SLNs were loaded with labeled etoposide and administered intravenously, and their biodistribution profiles were compared to free drug.^[333] Results showed that the positively-charged SLNs achieved a high plasma concentration and extended blood residence time, while both negatively- and positively-charged particles showed lower uptake by major RES organs compared to free etoposide.^[333] In addition, the positively-charged SLNs demonstrated maximal brain uptake (14-fold higher brain drug accumulation compared to negatively-charged SLNs and free etoposide at 4 hours post-administration).^[333] However, a separate study performed in rats suggests that this increased brain uptake comes at the expense of potentially significant BBB disruption.^[334] Neutral, negatively-charged and positively-charged SLNs were studied for their effects on BBB permeability, and the results indicated that although neutral SLNs and low-dose anionic SLNs ($10 \mu\text{g mL}^{-1}$) were safe for brain drug delivery applications, higher concentrations of both anionic and cationic SLNs ($20 \mu\text{g mL}^{-1}$) were detrimental to BBB integrity.^[334]

Polymeric NPs: Homopolymers and (amphiphilic block) copolymers can be used for the fabrication of a variety of polymeric nanoparticulate systems, including micelles, polymersomes/vesicles, polyplexes, nanocapsules and nanospheres.^[335] Some important members of these groups that have been applied in the context of brain drug-delivery are introduced below.

Micelles

Micelles are aggregates of amphiphilic surfactant molecules dispersed in aqueous solution. Typically, they form spherical structures, with hydrophilic “head” regions on the surface in contact with the surrounding solvent, and hydrophobic “tail” regions on the inside.^[336] While micelles are naturally formed from molecules or ions in bulk solution, polymeric micelles are self-assembled polymer shells composed of block copolymer macromolecules, with common ones being poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) and PEG-poly(caprolactone) (PEG-PCL).^[337] Polymeric micelles can have considerable stability, high loading efficiency, and sustained drug release profiles. They can also increase the solubility and bioavailability of poorly soluble drugs.^[337] In studies conducted by Liu et al., spherical micelles assembled from cholesterol-conjugated PEG and anchored with transcriptional activator (TAT) peptide (TAT-PEG-b-Col) were found to successfully cross the BBB in human astrocyte cell culture and in rat models.^[338, 339] The micelles were deliberately fabricated with average size smaller than 200 nm to facilitate brain uptake—although this introduced a drawback of low initial drug loading capacity—and demonstrated sustained drug release profiles over the course of 5–6 hours.^[338, 339] Results indicated that selective brain penetration was achievable as a result of the TAT peptide, which likely initiated AMT across the BBB and thereby enabled the micelles to localize both in astrocytes and around the cell nucleus of neurons.^[338, 339] In another study, small polymeric micelles (average size ~25 nm) modified with angiopep-2 were found capable of transporting amphotericin B (AmB), an antifungal agent that by itself demonstrates poor brain penetration, across the BBB in both rat and mouse models, as well as in cell culture.^[340] Results showed that usage of this drug carrier provided three primary benefits. First, the micelles, on account of their inherent aqueous stability and hydrophobic core, were able to improve the solubilization of AmB, an otherwise poorly water-soluble drug.^[340] Second, functionalization of the micelles with increasing amounts of angiopep-2 (up to 20 mol% of total polymers) enhanced both the transport of AmB across the BBB (brain

uptake of functionalized micelles was 1.6 fold higher than uncoated micelles and 3 fold higher than free drug), and the penetration of AmB into parenchymal tissues (functionalized micelles were found to localize in the brain's cortical layer, caudate putamen, hippocampus and substantia nigra, while plain micelles only accumulated in the cortical layer and caudate putamen). Increased brain uptake was attributed to RMT, since angiopep-2 is known to mediate transcytosis across the BBB via LRP-1 binding.^[340, 341] Third, usage of the micelles as drug delivery vehicles reduced the systemic toxicity of AmB towards mammalian cells.^[340] In the mouse biodistribution study, micelles (both plain and functionalized) containing AmB demonstrated much lower affinity for the liver and spleen than free drug itself, resulting in prolonged circulation times. Furthermore, incorporation of AmB into micelles resulted in a noticeable reduction in cytotoxicity and hemolysis (this was even more pronounced after functionalization with angiopep-2). In cell culture, BMECs remained almost 100% viable and demonstrated little to no hemolysis even at high micellar concentrations (50–100 µg AmB per mL).^[340] The reason for this reduction in toxicity was suggested to be due to the slow release profile and aggregation state of AmB. In its monomeric, non-aggregated form, AmB is reportedly non-toxic towards mammalian cells; however, in its aggregated form it interacts nonspecifically with both mammalian and fungal cells, thus causing widespread toxicity.^[342] Micellar drug loading may result in the selective release of monomeric, non-aggregated AmB, thereby improving its toxicity profile compared to free drug.^[340]

Dendrimers

Usually composed of poly(amidoamine) (PAMAM), dendrimers are three-dimensional, repetitively branched polymers that adopt a spheroidal and symmetrical morphology in water.^[40] They comprise three domains: (i) a multivalent surface, containing several potentially reactive functional groups; (ii) radially concentric interior shells, resembling tree-like branching from the core (defined by dendrons); and (iii) the core itself, to which dendrons

attach via focal points.^[16] The molecular structure is tightly packed at the periphery but loosely packed in the core, leaving spaces that facilitate drug entrapment.^[343] Drug molecules can associate with dendrimers in three ways: first, they can be covalently attached to the peripheries, forming dendrimer prodrugs; second, they can interact with the outer functional groups via ionic interactions; and third, they can be encapsulated within dendrimers via formation of dendrimer-drug supramolecular assemblies.^[344] Many of the physicochemical characteristics that define dendrimers make them useful for drug delivery purposes, including their monodispersity, water solubility, low toxicity, high loading capacity and large number of modifiable surface groups.^[40] The versatility of these NPs allows them to be modulated based on the environment; hydrophilic end groups can render a dendrimer with a hydrophobic core water-soluble, while hydrophobic peripheral moieties can render a dendrimer with a hydrophilic core lipid-soluble.^[16]

Functionalization of PAMAM dendrimers has proven to be an effective method of increasing penetration of cells in the NVU, both via neurosurgical and intravenous administration.^[345, 346] In a study conducted by Ke et al., PAMAM dendrimers were modified with angiopep (a ligand that targets LRP-1) and then complexed with DNA, yielding PAMAM-PEG-Angiopep/DNA NPs. After intravenous administration, these angiopep-modified NPs were found to be successful in crossing the murine BBB via clathrin- and caveolae-mediated endocytosis, as well as via partial macropinocytosis.^[346] Due to the presence of angiopep, interaction with LRP-1 was suspected to be the primary mechanism of cellular uptake.^[346] Although these angiopep-functionalized NPs were able to achieve greater passage across the BBB than uncoated NPs—for NPs incorporated with the maximal amount of angiopep, brain uptake was 0.25% of the injected dose versus 0.03% for uncoated NPs—one drawback associated with this pathway involved competition with endogenous ligands for the LRP-mediated transport system.^[346] In vitro tests showed that introduction of receptor-

associated protein (RAP), another endogenous ligand of LRP-1, caused a reduction in transport efficiency of the angiopep-functionalized NPs. Nevertheless, brain uptake was able to be enhanced by increasing the ratio of angiopep incorporated in the NP formulation. Another drawback was the significant accumulation of NPs in the kidney (almost 20% of the injected dose), a property that remained relatively unchanged despite variations in the angiopep ratio. This was deemed to be a property of the PAMAM dendrimer itself, since PAMAM is typically eliminated via the kidney.^[346] Dendrimers have also been successfully functionalized with other targeting ligands for transcytosis across the BBB, including lactoferrin^[347] and transferrin;^[348] both of these formulations were found to demonstrate higher BBB crossing ability than their unmodified counterparts, but incurred similar challenges to the aforementioned angiopep-functionalized dendrimers.

Nanospheres and Nanocapsules

Polymeric nanospheres are composed of a dense polymer matrix that enables the dispersion, adsorption, or binding of drugs. In contrast, polymeric nanocapsules consist of a core-shell arrangement—the shell is typically polymeric (although lipid nanocapsules do exist) and encapsulates an inner oily or aqueous core. Both types of NPs are currently being investigated for their potential utility in brain drug delivery applications. Chitosan, PLGA/PLA and poly(alkyl cyanoacrylate) (PACA) are among the most important polymers used in biomedical applications, and these are to be discussed below.

Chitosan. Chitosan is a biocompatible and biodegradable polymer that can function as a standalone NP or alternatively as a coating agent for NPs made of other materials (**Figure 30**).^[40] As a cationic polysaccharide, the molecular structure of chitosan contains free amino groups that render it insoluble in neutral or basic pH conditions. Under acidic conditions, however, the free amino groups undergo protonation, making chitosan selectively soluble in water.^[350] One potential application of this chemical property includes a drug delivery system

that can maintain its integrity in neutral and basic environments, but solubilize and degrade in acidic environments, thereby releasing the drug to the target region (tumor tissues, for example, have been observed to undergo significant pH changes).^[351, 352] There have already been a number of attempts to incorporate this stimuli-responsiveness into NP drug formulations, although the implications for brain drug delivery still have yet to be fully explored.^[353-355] Benefits associated with chitosan NPs include their controllable drug release profiles, their linear polyamine structure featuring free amine groups capable of cross-linking, their biocompatibility with living tissues (since, in individuals without shellfish allergies, they degrade slowly into harmless, absorbable products),^[356] and their mucoadhesive nature, which increases residual time at absorption sites.^[350] Chitosan NPs loaded with neuroactive compounds have demonstrated efficacy in crossing the BBB and acting therapeutically at target sites after intravenous administration, especially when surface-modified with protective and targeting moieties.^[357] In a study by Yemişçi et al., chitosan NPs were loaded with caspase-3 inhibitor—caspase-3 is a protease enzyme that promotes apoptosis, and it plays an important role in mediating neuronal cell death following ischemia—and functionalized with PEG to increase NP circulation time. Anti-TfR mAbs were subsequently conjugated to the ends of the PEG chains via biotin-streptavidin binding in an effort to promote increased passage across the BBB. Results in mice showed that NPs conjugated with anti-TfR mAbs were able to penetrate the brain, including both hemispheres, to a much greater extent, and for much longer, than those NPs lacking the targeting ligand. Functionalized NPs achieved maximum brain penetration at 75 minutes post-injection and sustained a fluorescence signal in the brain for 3 hours, whereas uncoated NPs failed to cross the BBB in detectable quantities.^[357] As a result of this, caspase-3 activity was significantly inhibited and neuroprotective effects were evident in mice treated with surface-modified NPs containing a

high-dose of drug, whereas empty NPs and those lacking the anti-TfR mAb were ineffective at reducing caspase-3 activity.^[357]

Due to their native cationic surface charge, non-surface modified chitosan NPs have also been shown to induce transport across the BBB, most likely as a result of AMT;^[358] this process is triggered by the electrostatic interaction between the positive NP surface and the negatively charged plasma membranes. Wang et al. synthesized *N*-Trimethyl chitosan (TMC) NPs and examined their ability to transport anti-neuroexcitation peptide (ANEP), a potential therapeutic for conditions such as epilepsy, across the BBB.^[358] The optimal NP formulation was found to have a degree of TMC quaternization of ~35%, encapsulation efficiency of ~80% (measured as the percentage of ANEP incorporated into NPs relative to the total amount added), loading capacity of 185 $\mu\text{g mL}^{-1}$, particle size of ~250 nm, and zeta potential of ~30 mV.^[358] In mice studies, these properties rendered the TMC NPs more capable of localizing in brain tissues after intravenous injection than free drug alone (the max brain concentration of ANEP delivered by NP formulation was 1.3 μg per mL of brain tissue, compared with 0.6 $\mu\text{g mL}^{-1}$ for free drug). However, one considerable drawback associated with the use of this nanoparticulate system was its unfavorable systemic biodistribution profile. After administering ANEP via the NP formulation, significant quantities of drug were detected in the heart, liver, spleen and kidney (maximum tissue concentrations were 6 $\mu\text{g mL}^{-1}$, 28 $\mu\text{g mL}^{-1}$, 1.4 $\mu\text{g mL}^{-1}$ and 8.3 $\mu\text{g mL}^{-1}$, respectively). These quantities were all greater than those measured after injection of free drug solution, indicating that the TMC NPs were responsible for causing non-discriminately increased drug uptake in off-target organs.^[358]

Intranasal delivery may offer an alternate, safer, quicker and more effective pathway by which chitosan NPs can be delivered to the brain.^[359, 360] In studies of both rat and mouse models, chitosan NPs have been found to accumulate in the brain to a greater extent and within a shorter timeframe via intranasal delivery than via intravenous injection (e.g., for

estradiol-loaded chitosan NPs, the maximum concentration of NPs per volume of CSF was found to be $\sim 75 \text{ ng mL}^{-1}$ nearly 30 minutes after intranasal administration, whereas a maximum CSF concentration of $\sim 30 \text{ ng mL}^{-1}$ was measured 60 minutes following intravenous administration).^[360] Similar observations were noted for rivastigmine-loaded chitosan NPs.^[359] These superior results for intranasal administration of NPs were achieved while maintaining lower levels of non-target drug dispersion (e.g. in liver and lungs) than observed via intravenous injection of NPs or even intranasal delivery of free drug.^[359, 360]

Poly(lactic-co-glycolic acid) (PLGA). PLGA is one of the most popular materials used in the formulation of NPs. It is a copolymer that exhibits biocompatibility, biodegradability and controlled drug release properties in vivo.^[40] The release kinetics of PLGA NPs can be modulated by varying the lactic acid to glycolic acid molar ratio and polymer molecular mass (for intracranial applications, PLGA NPs are reported to have better release kinetics than liposomes and micelles).^[9, 155] Drug entrapment efficiency in PLGA NPs depends on a number of factors, including solid-state drug solubility, molecular weight, drug-polymer interaction, and the presence of surface functional groups. Since PLGA is a hydrophobic copolymer, lipophilic drugs are easier to formulate in the dissolved state than hydrophilic drugs. The average entrapment amount typically ranges from 5–10% (wt/wt),^[15, 361] although this can vary substantially depending upon the property of the drug and the preparation procedure (in some instances, drug content can constitute up to 50% by weight of the NP formulation).^[362] PLGA NPs generally measure up to 200 nm in diameter and, just like many other NPs, can be modified with protective and brain-targeting moieties to enhance BBB uptake. Without surface modification, a large proportion of PLGA NPs are typically trafficked to systemic organs; one study found that after oral administration in mice, around 40% of injected NPs accumulated in the liver as a result of the RES, while another 25% localized in the kidney.^[363] Indeed, the RES is so highly effective that some unmodified NPs

have been known to display blood half-lives of only 2-3 minutes.^[15, 364] Fortunately, even with the high rates of PLGA accumulation in systemic organs, these NPs tend to exhibit little or no toxic effects both in vitro and in vivo.^[363]

Current challenges associated with PLGA NPs include irreversible adsorption of proteins to the polymer matrix (this is known as the “protein corona”,^[365] and is not unique to PLGA NPs), as well as inactivation of proteins during preparation, storage and administration.^[366] After functionalization, PLGA NPs have been successfully and safely used to deliver various drugs into the brain via multiple routes of administration, even intratympanic delivery.^[165] In one study, PLGA NPs were formulated with either of two stabilizers, PVA or human serum albumin (HSA), loaded with either of two model drugs, doxorubicin or loperamide, and coated with either of two surfactants, poloxamer 188 or PS 80.^[367] The doxorubicin-loaded NPs were subsequently investigated for their potential to exert anti-tumor effects in glioblastoma-affected rats, while the loperamide-loaded NPs were investigated for their potential to induce central analgesic effects in mice. In the case of both drugs, results indicated that PVA was the optimal NP stabilizer and that functionalization with either poloxamer 188 or PS 80 enabled significant drug passage across the BBB compared to uncoated NPs that were incapable of achieving transit.^[367] However, the poloxamer 188-coated NPs were found to induce greater and longer lasting anti-tumor effects in rats and antinociceptive effects in mice than the PS 80-coated NPs. In the glioblastoma-affected rats, coating with poloxamer 188 enabled long-term remission (>100 days tumor-free) in 40% of animals (4 out of 10), whereas coating with PS 80 yielded only one long-term survivor (the rest died before day 40).^[367] In mice, analgesic effects were measured as a percentage of the maximal possible effect using the tail-flick test. For the poloxamer 188-coated NPs, analgesic effects peaked at 80% of the maximum 15 minutes post-injection, were sustained at or above 70% for 90 minutes, and remained detectable for 120 minutes; meanwhile, for PS 80-coated

NPs, the analgesic effects peaked similarly at 15 minutes, before declining rapidly to 40% at 60 minutes.^[367]

Interestingly, it was found that the efficacy of the surfactants could vary depending upon the type of NP used. In analogous experiments conducted with poly(butyl cyanoacrylate) (PBCA) NPs (discussed in greater detail below), both PS 80 and poloxamer 188 induced similar, distinctive pharmacological effects when delivering loperamide^[368, 369] and doxorubicin^[370, 371] across the BBB (i.e. both surfactants were effective). This finding suggests that the core properties of a polymer material can influence the therapeutic efficacy of surface functionalization strategies. Also striking was the fact that upon comparing pharmacological profiles for PBCA and PLGA formulations, PBCA was suggested to be a faster degraded material since the effects of loperamide administration were sustained for a much shorter period than when PLGA NPs were used.^[367]

Poly(alkyl Cyanoacrylate) (PACA). PACA NPs are synthesized from alkyl cyanoacrylate monomers. These monomers, known for their highly reactive and adhesive properties, have found wide-ranging applications in industry, including in superglue and surgical glue (e.g., for the closure of skin wounds).^[372] In their polymeric form, however, PACA NPs have found application as a reliable carrier of cytostatics, antibiotics, antiviral agents, anti-fungal drugs and non-steroidal anti-inflammatory drugs, among others.^[373] Mechanisms and rates of drug release depend upon the type of drug loading in PACA NPs. Adsorbed drugs are released by desorption, while entrapped drugs are released by diffusion. The greater the affinity of the drug for the polymer, the slower the rate of release as the NP degrades (one prominent mechanism of PACA degradation involves hydrolysis of side chain ester bonds).^[372, 373] In their uncoated form, PACA NPs take on a relatively hydrophobic surface chemistry, thereby attracting greater amounts of protein adsorption and triggering faster uptake by the RES.²⁰² They are also generally incapable of crossing the BBB and

localizing in parenchymal tissues, instead achieving high tissue concentrations in systemic organs such as the liver and spleen.^[374] Functionalization with “stealth” and targeting moieties, however, can confer “stealth” and targeting properties to these PACA NPs, rendering them capable of circulating for longer periods and targeting different cell types more specifically.^[375] In the context of brain drug delivery, surface-modified PBCA NPs have been shown to successfully deliver drugs including loperamide,^[369] doxorubicin^[370, 371] and dalargin^[376] across the BBB in therapeutically significant quantities. In a number of studies, optimal NP circulation times and maximal therapeutic effects were observed for PBCA NPs coated with PS 80 surfactant,^[372, 374, 377, 378] although PEG has also been found quite useful in preventing phagocytosis and promoting NP circulation.^[379, 380] For example, PBCA NPs coated with PS 80 were found to be most effective at delivering dalargin to the brain after intravenous injection; this conclusion was reached after comparing the maximum analgesic effects exerted by various surface-modified PBCA NP formulations.^[378] In another study, PBCA NPs coated with 1% PS 80 demonstrated a significant increase in the uptake of rivastigmine (a reversible cholinesterase inhibitor) into the AD-affected brain compared to free drug.^[381] Gao and Jiang observed that among four different size ranges of tested PBCA NP formulations, PS 80-coated NPs with size below 100 nm achieved the greatest brain concentrations of immunosuppressant drug methotrexate after intravenous injection in rats (70 nm NPs achieved maximum brain tissue concentrations of 95.36 ng g⁻¹ in the cerebrum and 82 ng g⁻¹ in the cerebellum).^[374] It is suggested that PACA NPs functionalized with PS 80 may achieve transport across the BBB via the RMT pathway, thereby explaining the superior efficacy of PS 80 as a coating surfactant (PS 80 may interact with lipoprotein receptors on the luminal side of the BBB to induce transcytosis).^[371, 380, 382] However, imaging of fluorescently tagged NPs assembled from amphiphilic PACA copolymers suggested that in this case, transport across the BBB occurred predominantly via the AMT pathway.^[383] The

aforementioned findings are congruent with each other, and suggest that the specific mode of BBB uptake depends upon the relevant properties of the NP formulation—a positively charged NP may initiate AMT, while an appropriate surfactant-coated NP may initiate RMT.

One potential drawback associated with PACA NPs is their variable, drug-dependent encapsulation efficiency.^[374] Although some drugs such as doxorubicin, paclitaxel and dalargin demonstrate a high affinity for PACA leading to a high encapsulation efficiency (for example, up to 80–90% encapsulation efficiency for paclitaxel-loaded PBCA NPs, defined as the percentage of drug incorporated into NPs relative to the total amount of drug added),^[384, 385] others including methotrexate demonstrate a much lower encapsulation efficiency (40% in the case of methotrexate-loaded PBCA NPs), causing suboptimal drug accumulation in the brain and consequently unremarkable therapeutic results.^[374] Another challenge identified by Gao and Jiang is that even after crossing the BBB with the help of nanoparticulate carriers, hydrophilic drugs such as methotrexate have a tendency to accumulate predominantly in the CSF rather than the parenchymal tissues, thus limiting the efficacy of treatment.^[374]

Nevertheless, PACA NPs often appear to fulfill key requirements for a CNS drug delivery system: ease of preparation and storage, generally adequate drug loading capacity (in the case of rivastigmine loaded into PBCA NPs, loading capacity ranged between 9.5–18% depending upon the drug polymer ratio),^[381] suitable biodegradability, limited in vivo toxicity, and viability for scale-up of production.^[372]

Inorganic NPs: Many inorganic NPs exhibit unique physical properties that can be harnessed for brain drug delivery applications. Among these are gold NPs (AuNPs), magnetic NPs (MNPs), ceramic NPs, fluorescent nanodiamonds (FNDs), and upconversion NPs (UCNPs), all of which are discussed below.

Gold NPs (AuNPs)

AuNPs have the potential to become very useful drug delivery vehicles on the basis of their unique physicochemical properties including ultra-small size, large surface area-to-mass ratio and ease of functionalization.^[16] In addition, AuNPs possess optical properties that many other types of NPs do not. Depending upon their size and shape, AuNPs can strongly adsorb or scatter incident light at a certain resonance wavelength. This is known as localized surface plasmon resonance (LSPR) (**Figure 31**).^[386] As the refractive index near the gold surface increases, the NP's LSPR shifts to longer wavelengths. LSPR peaks can be specifically tuned into the near infrared region (800–1100 nm), which is an optically transparent window for soft tissues.^[387] Due to the nanoscale biosensing capability that this optical property entails, AuNPs could feasibly be used as theranostic agents—combining therapeutic and diagnostic functionality. AuNPs can also convert near IR light into heat via the photothermal effect, potentially leading to applications in cancer therapy.^[388, 389] In a study conducted by Sonovane et al., uncoated AuNPs of various sizes (15, 50, 100 and 200 nm) were formulated and administered intravenously in mice to determine their size-dependent biodistribution properties.^[390] Tissue analysis revealed that AuNPs with a size of 15 nm or 50 nm were able to cross the BBB and accumulate in the brain most effectively (100 nm AuNPs accumulated in the brain to a lesser extent, and 200 nm AuNPs were only present in trace amounts). The total number of AuNPs that entered the brain was found to be inversely dependent upon size, and therefore greater for 15 nm particles than for 50 nm particles. However, the total collective volume of gold (proportional to the total volume of AuNPs) that entered the brain was found to be similar for both particle sizes.^[11, 390] In separate organ distribution studies, the ability of uncoated AuNPs to penetrate the BBB was found to be a size-dependent phenomenon, with AuNPs below a threshold of 20 nm achieving uptake into the brain.^[391, 392] In addition, however, intravenous administration of AuNPs of various sizes (10, 50, 100 and 250 nm) in rats was shown to cause significant off-target distribution to systemic organs, with

much of the NP dose accumulating in the liver (20–46% of administered dose) and spleen (1.2–2.2% of administered dose) after 24 hours and remaining present even after 2 months.^[391] Two factors that may contribute to these organ distribution patterns include the small AuNP sizes, resulting in passage through the fenestrated epithelia of systemic organs, and the uncoated nature of the AuNPs, resulting in sequestration by the RES.^[391] Studies of the accumulation patterns and toxic effects of uncoated AuNPs after repeated administration of varying doses in mice suggest that although AuNPs can experience significant distribution to systemic tissues, they do not typically produce cell mortality or any considerable toxic effects.^[393] However, this is a potentially variable phenomenon since toxicity depends upon the specific particle characteristics, including composition, size and surface properties. In the aforementioned studies, 12.5 nm AuNPs with spherical morphology and zeta potential of –53 mV were used.^[393] In other studies, AuNPs smaller than 4–5 nm have been proposed to potentially induce cellular toxicity as a result of penetration of the nuclear compartment and subsequent DNA binding.^[388]

Transport of uncoated AuNPs across the BBB is hypothesized to occur via active transport mechanisms, although further investigation is needed to elucidate the exact pathway of entry.^[393, 394] Regardless, a number of studies have shown that without surface modification, AuNPs achieve suboptimal levels of brain accumulation.^[391-393] Sousa et al. conducted a biodistribution study in mice, intravenously administering 15 nm AuNPs coated with oppositely charged polyelectrolytes polyallylamine hydrochloride (PAH) (a polycation) and polystyrenesulfonate (PSS) (a polyanion) as well as HSA.^[395] A natural component of blood, HSA was incorporated with the twofold purpose of neutralizing potential cytotoxic effects caused by the polyelectrolytes, and increasing circulation time of the AuNPs by limiting immune recognition. Both of these aims were achieved—no significant toxicity or barrier damage was detected in BMECs, and the circulation time of AuNPs was observed to

be significantly prolonged (up to 48 hours).^[395] An analysis of the subsequent brain distribution patterns revealed that AuNPs that crossed the BBB predominantly aggregated in the hippocampus, thalamus, hypothalamus and cerebral cortex, without entering the nucleus of the cells in the different regions.^[395, 396] AMT was proposed as the primary mode of transport, since the cationized form of HSA has been known to selectively induce transcytosis across the BBB.^[395, 397] These findings were of special interest, since the distribution patterns were observed to occur near regions affected by AD, PD and prion disease.^[395] Indeed, there is evidence that functionalization of AuNPs can result in drug systems that are water miscible, biocompatible, long circulating, protected against chemical degradation and more effectively targeted to the brain.^[398] Nevertheless, controversy still surrounds the suitability of AuNPs to biomedical applications involving the brain, suggesting that extensive nanotoxicology studies should be carried out on a case-by-case basis.^[399]

Magnetic NPs (MNPs)

Core-shell MNPs are composed of an inorganic magnetic core NP surrounded by a biocompatible shell coating that provides stabilization under physiological conditions.^[280] Without any surface coatings, many magnetic particles have hydrophobic surfaces with large surface area-to-volume ratios, which may cause particle agglomeration and the formation of large clusters. Not only can this aggregation reduce the intrinsic magnetic properties possessed by the particles, but it may also trigger opsonization and immune cell uptake.^[16]

In composite form, however, MNPs demonstrate considerable advantages. Their magnetic properties can render them theranostic agents, since they can serve as both MRI contrast agents and therapeutic delivery vehicles that have the ability to be targeted by magnetic attraction (e.g., in one study, magnetic liposomes were found to achieve a 10-fold increase in brain levels compared to non-magnetic NPs when a local magnetic field was applied).^[199, 280, 400] The core itself can be made up of two types of magnetic materials—

paramagnetic and superparamagnetic materials. Although both of these exhibit similar properties, they are mainly differentiated by the value of their magnetic moment, which is markedly higher for superparamagnetic materials. The ultimate significance of this is that unlike paramagnetic agents, superparamagnetic agents exhibit no magnetic properties outside an external magnetic field.^[401] Thus, superparamagnetic agents are often more useful in biomedical applications, since this lack of magnetization can make it easier for them to avoid aggregation and maintain their colloidal stability.^[280]

The core NP can be composed of various materials. The most commonly used is iron oxide, which is found in both magnetite (Fe_3O_4) and maghemite ($\gamma\text{Fe}_2\text{O}_3$) forms, with magnetite being the more commonly used. Iron oxide NPs are typically spherical and have diameters between 10–100 nm.^[388] Hypothetically, they could be broken down naturally, resulting in the release of ferric iron that could be added to the body's stores and eventually used by red blood cells as hemoglobin.^[388] In practice, however, iron oxide NPs can pose a challenge in terms of efficient clearance due to their small size and propensity to reach high local concentrations within cells.^[280, 388] They are believed to exhibit low toxicity in the CNS,^[402, 403] but any impurities that accumulate during production, including metal ions or organic stabilizers, can diminish their viability and lead to potentially cytotoxic effects in vivo.^[388] Although iron oxide is a ferromagnetic material, it becomes superparamagnetic when its size is reduced below 12–15 nm.^[388] Superparamagnetic iron oxide (SPIO) NPs have a total size (iron oxide core plus coating) greater than 50 nm, whereas ultrasmall superparamagnetic iron oxide (USPIO) NPs have a size below 50 nm.^[401] Based on biodistribution studies, it is hypothesized that to maximize cellular internalization efficiency and minimize cytotoxicity, the ideal size range for fully coated iron oxide NPs is between 10–30 nm.^[388]

Other types of core materials that can be used include metals (such as iron, cobalt and nickel), although these are often chemically reactive—they tend to form oxides in the presence of water and oxygen—and may therefore need to be protected by external coatings, and metal alloys (such as iron-platinum (Fe-Pt)) that tend to have greater chemical stability but also greater magnetization, thus often requiring protective coatings to prevent oxidation and corrosion.^[280] Various options also exist for the shell coating that encases the core magnetic NP. Polymeric coatings provide a steric barrier to prevent NP agglomeration and avoid opsonization (**Figure 32**). Liposomes and micelles can encapsulate magnetic NPs and deliver them to the target site. Inert inorganic coatings, such as gold and silica, can protect against chemical degradation and prevent the release of potentially toxic compounds (**Figure 33**).^[280] Surface modifications can be made to improve the pharmacokinetic profile of MNPs in vivo. Protective and targeting ligands can be conjugated covalently or non-covalently, and varying degrees of surface charge can be incorporated into the NP structure. Strongly anionic particles may result in faster opsonization and increased liver uptake.^[280] Cationic particles, on the other hand, may in some applications be less stable and exert the greater cytotoxic effects.^[388] In an effort to determine the optimal tradeoff between toxicity and internalization efficiency, Soenen et al. analyzed the uptake and cytotoxicity profiles of magnetoliposomes incorporated with varying amounts of positively charged surfactant (DOTAP and its distearoyl analogue DSTAP). They found that when magnetoliposomes were coated with an outer shell containing ~3% weight positively charged DSTAP surfactant, cellular uptake was substantially increased whilst cell viability remained uniform.^[406]

MNPs have been tested in a number of different animal models of neurological disease, but one recent area of focus has been the treatment of brain tumors.^[389] In a study by Chertok et al., MNPs with an iron oxide core and starch shell, measuring 110 nm in diameter, were injected intravenously in rats bearing gliosarcomas and investigated for their ability to

achieve magnetically-targeted localization in the diseased regions (the magnetic field was applied for 30 minutes).^[407] Using minimally-invasive MRI monitoring, the magnetically-targeted MNPs were found to achieve a 5-fold greater exposure to tumor tissues than untargeted MNPs.^[407] Additionally, the increased accumulation of MNPs in diseased brain regions was not accompanied by an equivalent increase in NP exposure to the contra-lateral, normal brain (there was a 9.5-fold difference in NP concentrations between tumor tissue and the contra-lateral normal brain).^[407] This phenomenon was unique to magnetically-guided NPs, and a similar degree of disease selectivity was not observed in untargeted NPs. Another key finding was that after being guided to target regions, MNPs were retained in glioma tissues long after the magnetic field was removed (approximately 100 minutes post-removal). Again, this was in stark contrast to the untargeted NPs. An 11.5-fold higher NP concentration was found in excised tumor tissues of targeted animals compared with untargeted animals approximately 50 minutes after injection.^[407] Finally, both preclinical and clinical trials with the same NP formulation as used in the aforementioned study suggested that systemic administration of these MNPs induced no toxic effects and was well tolerated, even at high doses.^[408, 409] Collectively, these findings illustrate the merits of magnetically-guided drug targeting and point to the potential utility of this strategy in precisely treating various neurological diseases.

Other Inorganic NPs

Several other inorganic materials have been used as the basis for CNS drug delivery applications, and some of these demonstrate uniquely desirable properties. Ceramic NPs, for example, typically have an inherently porous nature and small size, and show negligible signs of swelling or other structural changes with pH.^[16] Fluorescent nanodiamonds (FNDs) are chemically inert carbon-derived particles that exhibit biocompatibility, prolonged photostability (can be tracked after 7 days using confocal microscopy and flow cytometry),

negligible toxicity, and intrinsic fluorescence.^[410] A relatively novel technology, FNDs possess innate neuroprotective properties, can be conjugated with various drugs to increase drug retention and targeting,^[410] and can also function as a versatile tool for long-term cell tracking, super-resolution imaging and nanoscale temperature sensing.^[411] Upconversion NPs (UCNPs) constitute another unique type of inorganic particle, and are composed of nanoscale crystals doped with trivalent lanthanide ions (e.g. Yb³⁺, Er³⁺) dispersed in a dielectric host lattice.^[410] They possess unique optical features, including MRI and upconversion luminescence (UCL) imaging, and can function as neuroprotective drug delivery vehicles. In an effort to leverage this unique bimodal imaging feature (both MRI and UCL imaging) of UCNPs, Ni et al. developed nanoprobes comprising UCNPs functionalized with PEG and covalently coupled with angiopep-2.^[412] After intravenous administration in glioblastoma-bearing mice, these nanoprobes were observed to cross the BBB via RMT, before localizing in glioblastoma tissues via similar angiopep-2-mediated transport.^[412] Follow up toxicity studies revealed no appreciable adverse effects in either systemic or central organs as a result of administering the UCNP-based formulation.^[412] The targeting efficacy of these nanoprobes, coupled with their unique optical properties, rendered them significantly more effective at imaging tumor sites than the single-mode imaging agents Gd-DTPA (an MRI contrast agent) and 5-aminolevulinic acid (a fluorescent dye), both of which are currently used in clinical practice.^[412] These results suggest that usage of the UCNP-based imaging agent has the potential to improve preoperative tumor diagnosis and intraoperative surgical positioning, thereby resulting in more accurate surgical resection of cancerous tissues.^[412] Another separate but also potentially useful application of these nanoprobes includes tumor radiotherapy, since UCNPs with high zeta potential have elsewhere been identified as effective radiosensitizers.^[413]

5.3.2. NP Surface Property Modifications

Although NPs can afford therapeutic agents with a safer and more effective means of distribution, there are three main challenges that hinder their in vivo efficacy (see **Table 4** for summary).

Table 4. A summary of the main challenges facing NP-based drug delivery in vivo, and the relevant surface property modifications that can be made to overcome these challenges

Challenge	Surface property modification	References
Tendency of NPs to aggregate during formulation, storage and application	Modification with stabilizing coatings; cryoprotection (e.g., treatment with trehalose)	[155]
Formation of protein corona, opsonization, and uptake by the RES	Coating with poly(ethylene glycol) (PEG), poloxamine 908, polysorbate 80 (PS 80), dextrin, hyaluronic acid, prostate-specific antigen (PSA), N-(2-hydroxypropyl) methacrylamide (HPMA) or other surfactants, or exploit the protein corona formation.	[15, 16, 280, 414-416]
Intracellular trafficking to acidic lysosomal compartment	Cationic NPs, NPs functionalized with pH-sensitive ligands or ligands with intrinsic lysosomal escape mechanisms (including FC5 sdAb and ligands against DT _R)	[184, 199, 278, 417]

Aggregation: The first challenge is that NPs have a tendency to aggregate during formulation, storage and application due to their large surface area-to-volume ratio. This can occur due to their own inherent chemical properties, or due to external factors. For example, during

preparation, some NPs undergo lyophilization—also known as freeze-drying. The purpose of this is to stabilize NPs for long-term storage, after which they can be reconstituted into their original form for administration.^[418] However, this can also cause the NPs to aggregate, making it difficult to redisperse them in aqueous solution.^[155] Trehalose is a natural, alpha-linked, non-reducing disaccharide that can function as a cryoprotectant—its purpose is to reduce the aggregation of NPs and enhance their separation without impacting on size, morphology or yield.^[155] When treated with trehalose, small NPs have demonstrated better penetration of the brain parenchyma via intracranial CED than those lacking cryoprotection.^[155]

Uptake by RES: After systemic administration, a substantial challenge to effective drug delivery can be the RES. As NPs circulate through the vasculature, nonspecific interactions occur between their shells and the many classes of proteins residing in the bloodstream. This can result in the adsorption of opsonins—including complement proteins, apolipoproteins, fibronectin, and antibodies—on their surface, forming a biomolecular “corona”.^[419-422] The corona can act as a signal to nearby immune cells, including monocytes and macrophages, which can interact with the opsonins via their membrane receptors and subsequently induce phagocytosis and NP sequestration.^[419-422] This process, by which NPs are targeted for degradation, is known as opsonization (**Figure 34**).^[423]

The RES has been observed to be especially effective at clearing cationic NPs from circulation, with some NPs reported to have circulation half-lives of less than one minute.^[425, 426] Grislain et al. conducted a study of the tissue distribution, blood clearance and excretion of biodegradable cyanoacrylate NPs, and found that up to 80–85% of the original NP concentration ended up being removed from the vascular space within a very short timeframe, suggesting decreased drug exposure at the cerebrovasculature and limited penetration into the brain.^[415, 427] Based on in vitro studies, it is suggested that non-ionic, hydrophobic surfaces

actively promote protein adsorption, while negative surfaces are activators of the complement system.^[428]

Apart from engineering smaller sized NPs (between 10–100 nm),^[429] the primary strategy that is employed to overcome RES uptake involves coating NPs with hydrophilic surfactants.^[415] Since the 1970s, PEG, a largely inert hydrophilic polymer, has been used as a key defense mechanism for circulating NPs (**Figure 35**).^[430] PEGylation, as the process is dubbed, involves conjugating PEG chains to the surface of NPs via covalent attachment, physical entrapment or adsorption (**Figure 36A**).^[431] Alternatively, PEG can be incorporated into the molecular structure as a copolymer (e.g., PEG-PLA amphiphilic block copolymers).^[432] Depending upon their surface density, PEG blocks most commonly take on either a brush-like (elongated coil, high-density) conformation or a mushroom-like (random coil, low-density) conformation (**Figure 36B**).^[431, 433]

Studies have shown that longer and more densely packed PEG chains built out of brush-like monomers can be effective at preventing corona binding and reducing phagocytosis (**Figure 37**).^[434-437] In contrast, PEG chains with mushroom-like configuration have actually been shown capable of favoring RES uptake.^[437] In extending the Alexander-de Gennes model to NPs, the optimal distance between PEG chains should be around 1 nm to repel small globular proteins (of radius ~2 nm), and 1.5 nm to repel larger proteins (of radius 6-8 nm).^[436] Further quantitative studies suggest that PEG chains should have molecular weight of at least 5000 Da and be covalently linked to the NP surface to maintain sufficient stability and maximal anti-opsonic effects, whilst avoiding desorption or displacement in vivo (although NP coatings are not completely invulnerable to degradation under adverse biological conditions).^[436, 438] Although some sources propose that drug loading capacity and release rates can be lowered by the PEG moiety,^[199] others suggest that the PEG moiety has minimal or no effect on drug loading,^[362] so long as self-assembled PEG polymers are directly bound

to the NP surface (either covalently or non-covalently).^[439]

PEGylation has proven extremely successful in prolonging blood circulation, increasing serum stability, reducing immunogenicity and preventing interactions with non-target cells,^[440] but studies show that it may not be a successful brain targeting strategy^[441]—i.e., it can increase drug exposure to the cerebral capillaries but may not improve brain uptake (potentially due to the increased molecular weight and hydrophilicity).^[442, 443] To achieve the latter, specific receptor-targeting ligands can be covalently conjugated to the terminal amine or carboxyl groups of 1–2% of PEG chains.^[280] This approach, as opposed to direct conjugation with the NP surface, reduces steric hindrance imposed by the protruding PEG chains.^[444, 445] Yet, despite the perceived benefits associated with PEGylation, one potential drawback that has been identified is the induction of anti-PEG antibodies and complement activation.^[446, 447] Studies have shown that repeated injection of PEGylated therapeutics can result in unexpected immune-mediated side effects, with anti-PEG antibodies triggering complement activation and subsequent RES uptake.^[448]

Other surfactants have also been shown to exhibit similar NP protective effects. Poloxamine 908, when used as a surfactant on PACA NPs, has been shown in both mouse and rat models to significantly reduce RES uptake and liver accumulation compared with uncoated NPs, and simultaneously increase blood circulation times.^[449] Calvo et al. observed that 1 hour after intravenous administration in mice, only ~11% of the injected dose of uncoated PACA NPs remained in the blood, whereas ~52% accumulated in the liver. However, coating with poloxamine 908 significantly improved drug biodistribution properties, with ~40% of coated NPs remaining in the blood and only ~13% accumulating in the liver after 1 hour.^[449] Even more striking results were observed in rat models.^[449] Several other studies have confirmed that poloxamine 908 can improve the organ distribution and circulation time of intravenously injected colloidal carriers.^[450-453] However, one

contradictory study suggests that poloxamine-coated NPs *do* exhibit complement-activating nature. The authors of this study suggest instead that complement consumption is dependent upon the surface density and brush conformation of the poloxamine coating, with higher density coating correlating to suppressed complement consumption.^[454] PS 80 has been shown to be effective not only at reducing RES uptake, but also at mediating RMT into the brain (discussed further below).^[415] A rat model study conducted by Gulyaev et al. showed that the brain concentration of systemically administered doxorubicin could be increased 60-fold when delivered via PBCA NPs coated with PS 80 (instead of uncoated PBCA NPs).^[414] Another, separate rat model study suggested that PS 80-coated PBCA NPs with diameter below 100 nm were an ideal therapeutic candidate for overcoming the BBB, since they exhibited the greatest drug concentration within the brain.^[374] In preliminary studies conducted by Olivier et al., it was suggested that PS 80-coated NPs utilize a mechanism of entry into the brain that causes toxicity (as a result of non-specific permeabilization of the BBB),^[455] however, this was challenged by Kreuter et al., who showed that penetration of the BBB occurs via specific and non-toxic mechanisms.^[382] Dextrin, hyaluronic acid, prostate-specific antigen (PSA), *N*-(2-hydroxypropyl) methacrylamide (HPMA) and various other poloxamers, poloxamines and PEG derivatives have also been suggested as effective polymer surfactants with protective capabilities.^[9, 16]

Lysosomal Trafficking: The third challenge that NPs face is lysosomal trafficking. After undergoing receptor-mediated endocytosis, NPs are transported through the endothelium by induced vesicles. Instead of being routed directly from luminal to abluminal membrane, however, many vesicles are instead routed to the lysosomal compartment, before subsequently being degraded or recycled to the luminal membrane.^[229] The low pH environment of the lysosome triggers the activation of numerous hydrolytic enzymes that degrade NPs and thereby reduce their ability to enter the brain.^[187] NPs therefore need to bypass the

endosomal–lysosomal pathway to reach the brain. This is no small feat, and despite several attempts to engineer “smart” stimuli-responsive drug systems, no broadly applicable technology as yet exists.^[456] Nevertheless, it has been postulated that bypassing of the endosomal-lysosomal pathway can be achieved by the use of cationic NPs as well as NPs functionalized in other ways.^[187] Cationic NPs are most commonly composed of DOTAP mixed with DOPE.^[315] Alternatively, they can be built out of positively charged block copolymers such as PEI-PEG or polymers such as PAMAM dendrimers.^[199, 417] If the NPs themselves are not inherently cationic, a positive surface charge can also be established by coating them with polyamine functional groups.^[199] This positive charge is believed to protect NPs against lysosomal degradation, potentially as a result of the ‘proton sponge effect’, where excess amino groups on the NP surface buffer acidification of the endocytotic compartments.^[199] So-called fusogenic pH-sensitive NPs, which generally contain DOTAP and DOPE, undergo protonation of their titratable acidic groups upon acidification in the endosomal environment. This destabilizes the vesicle membrane and enables interaction with the opposite endosomal membrane, leading to lipid merging and potential membrane fusion.^[187] In this way, NPs bypass the lysosome and reach the abluminal membrane. However, some of the drawbacks associated with some types of cationic NPs include increased cytotoxicity, lower loading capacity, and diminished release rates.^[199]

Another method to bypass the lysosomal compartment includes functionalizing NPs with specific targeting ligands that have intrinsic lysosomal escape mechanisms, including the FC5 sdAb,^[278] and ligands for the DT_R.^[457] Ultimately, despite the challenges posed by unfavorable intracellular trafficking, RMT has proven successful in transporting NPs across the BBB in vivo in animal models, and it is believed that this success could feasibly be extended to human patients as well.^[192]

5.3.3. BBB Targeting Strategies

To derive an accurate understanding of the therapeutic efficacy of different CNS drugs, precise analytical methods for characterizing and quantifying nanomaterial transport within the body are invaluable. To this end, a number of in vitro and in vivo studies have been conducted, which aim to provide a quantitative analysis of NP transport both across the BBB and within the CNS.^[52, 173, 221, 395, 407, 414, 449] For in vivo studies, high-performance liquid chromatography assays can be used to determine drug concentrations in blood plasma and different tissues, thereby providing a measure of brain-targeting specificity for a given drug formulation.^[414] MRI is another versatile tool that has been used to visualize and quantify, among other things, the amount of NP accumulation in the brain,^[280] the amount of NP exposure to target regions,^[407] the therapeutic effect on disease targets—e.g., measurable reduction in tumor size^[322]—and the degree of BBB opening reversibility after transient disruption.^[221] Near-infrared time-domain imaging can be used to measure the general biodistribution of fluorescently labelled NPs after intravenous administration, although its spatial resolution presents a limitation in that it does not allow for the precise localization of NPs within the brain.^[395] To achieve this, complementary imaging methods such as X-ray microtomography, confocal laser scanning microscopy, and epifluorescence microscopy can be employed.^[395] These allow imaging of NPs at the cellular level and can reveal distribution patterns, as well as particular modes of transport through the BMECs (e.g., in a scan, the detection of NPs within vesicles may suggest either RMT or AMT).^[395] This section aims to explore various BBB-targeting strategies for NPs, with a focus on some of the engineering considerations that are requisite for optimizing CNS delivery while minimizing unwanted off-target effects.

Analogue-Based Drug Design for CMT: Despite the fact that CMT is a very selective and restrictive method of transport, NPs have been shown to successfully use carrier-mediated systems to cross the BBB. Surface functionalization of liposomes with α -mannose (but not β -

mannose) derivative facilitates CMT via the GLUT1 transporter.^[178] Meanwhile, 60-nm NPs coated with choline derivative have been found capable of crossing the BBB in vitro (via the cation transporter) at a faster rate than uncoated NPs.^[178] To study luminal-to-abluminal transport, NPs were labelled with fluorescein and added to the luminal chamber of an in vitro coculture of bovine brain capillary endothelial cells and rat astrocytes. After 4 h of incubation, fluorescence spectrometry and microscopy techniques were used to measure the differential transport characteristics of the NP systems across the cell monolayer. Yet despite these findings, further studies suggest that the stringent size and structural requirements imposed by BBB transporters make the CMT pathway better suited to free drug molecules than to NPs.^[177]

Table 5. A summary of RMT- and AMT-based therapeutic strategies for crossing the BBB

Receptor types	Functionalization with receptor-targeting ligands for RMT		References
	Targeting ligands	Examples (proof of concept)	
Transferrin receptor (TfR)	Endogenous Tf, mAbs against TfR (OX26, 8D3, RI7217)	<ul style="list-style-type: none"> • Loperamide-loaded HSA NPs with covalently-bound Tf or OX26 or RI7217 found to exert strong anti-nociceptive effects in the mouse brain. • In separate murine studies, 8D3 found to have greater brain uptake compared with RI7217 and OX26. RI7217 found to have greatest brain selectivity (not measurably taken up by liver). 	[54, 192, 388, 458-460]

<p>Insulin receptor (IR)</p>	<p>mAbs against IR</p>	<ul style="list-style-type: none"> • In a rhesus monkey study, genetically engineered HIRMAb (human insulin receptor MAb) was produced and found to have identical reactivity to the human BBB HIR as the original murine MAb, proving its efficacy at crossing the BBB in vivo. • In a separate study, humanized, murine-derived HIRMAb was found to accumulate in all parts of the Rhesus monkey brain after intravenous injection. 	<p>[146, 278, 461, 462]</p>
<p>Lipoprotein receptors (LDLR, LRP1, LRP2)</p>	<p>PS 80, ApoE, ApoB, Apo A-I, angiopep-2, lactoferrin (Lf), melanotransferrin (MTf), RAP, tPA, plasminogen activator inhibitor-1 (PAI-1), APP, heparin cofactor II, heat shock protein-96 (HSP-96), alpha-2-macroglobulin (α</p>	<ul style="list-style-type: none"> • In rat studies, Gulyaev et al. showed that brain concentrations of systemically administered doxorubicin could be increased 60-fold when delivered via PS 80-coated PBCA NPs instead of uncoated PBCA NPs. • Covalent linkage of ApoE to the surface of HSA NPs strongly enhanced the delivery of loperamide into the brain in mouse models. • In an in vitro BBB model, angiopep-2 	<p>[192, 194, 278, 341, 414, 460, 463, 464]</p>

	2M)	<p>exhibited higher transcytosis capacity and parenchymal accumulation than Tf, Lf and avidin. Comparative studies suggest that angiopep-2 may compete with α2M for LRP1-mediated transcytosis.</p> <ul style="list-style-type: none"> • In both in vivo mouse models and in vitro cell monolayers, RAP was shown to exhibit greater BBB permeability compared to both MTF and Tf. 	
Diphtheria toxin receptor (DT _R)	Exogenous DT, cross-reacting material 197 (CRM 197)	<ul style="list-style-type: none"> • In an in vitro BBB model, CRM197 shown to induce apical-to-basal transcytosis involving the caveolin-mediated pathway. • CRM197 conjugated to cargo of size 40kDa found to be taken up by the brain in vivo (in guinea pigs); significant accumulation was observed in the brain cortex, assumedly due to high expression of DT_R. 	[55, 465, 466]
Folate receptor (FR)	Folic acid	<ul style="list-style-type: none"> • Wu and Pardridge showed that folic acid derivatives can be taken up by 	[467]

		either the rat brain in vivo or by human BMECs in vitro, and thus concluded that a saturable folic acid transport system exists at the BBB.	
FC5 orphan receptor (TMEM30A)	FC5 sdAb	<ul style="list-style-type: none"> In a study conducted by Muruganandam et al., FC5 sdAbs and FC44 sdAbs were able to transmigrate across an in vitro model of the human BBB. 	[278, 468, 469]
Functionalization with cationic/amphiphilic constituents for AMT			
Strategies	Constituents	Examples (proof of concept)	References
Cationic or amphiphilic NPs	DOTAP + DOPE, cationic and amphiphilic block copolymers	<ul style="list-style-type: none"> Calvo et al. found that after intravenous administration, PEGylated poly(cyanoacrylate) NPs composed of amphiphilic block copolymers (PEG-PHDCA) were able to cross the murine and rat BBB and localize in the brain to a greater extent than non-amphiphilic PHDCA NPs (both uncoated and coated forms). The long-circulating property of amphiphilic NPs was thus validated in vivo. 	[199, 350, 417, 449]
Cationic ligands	CPPs (model	<ul style="list-style-type: none"> Both in vivo (rat model) and in 	[10, 195, 332,

	<p>amphipathic peptide (MAP), Antennapedia (Antp), transportan, penetratin, fusion sequence-based peptide (FBP), transactivator of transcription (TAT), protegrin-derived pegelin proteins (e.g., Syn-B1 and Syn-B3), trimethyl chitosan (TMC), PSS/PAH + HSA</p>	<p>vitro studies have shown that conjugation of polymeric core/shell NPs with TAT results in significantly increased uptake into the brain compared with free drug as well as NPs lacking the TAT peptide.</p> <ul style="list-style-type: none"> • TMC surface-modified PLGA NPs, when injected into the caudal vein of mice, were found to accumulate in the cortex, paracoele, third ventricle and choroid plexus epithelium to a much greater extent than NPs lacking TMC conjugation, without affecting cell viability. • Conducting both in vivo (murine model) and in vitro studies, Lu et al. found that PEG-PLA NPs covalently functionalized with cationic bovine serum albumin (CBSA) were able to cross the BBB via transcytosis to a much greater extent than native bovine serum albumin (BSA) conjugated NPs. 	<p>338, 358, 470-472]</p>
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		The CBSA-NPs did not impact the integrity of BMEC tight junctions, and exhibited little toxicity to the BBB.	
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Functionalization with Receptor-Targeting Ligands for RMT: RMT-based strategies involve conjugating targeting ligands to the NP exterior to facilitate uptake via specific BBB receptors. However, since many NPs are initially PEGylated to increase their blood circulation time, simply attaching these ligands to the NP surface can lead to steric hindrance and ineffective ligand–receptor binding. Targeting can be enhanced by covalently attaching ligands to the ends of the PEG chains (or other spacer molecules), such that they extend outside the dense protective corona.^[444, 445, 473] Attachment can be achieved by reacting the ligands with amine-reactive or thiol-reactive functional groups that occur at the ends of the PEG strands,^[474] although this is not the only strategy. For example, Howard et al. synthesized protein–polymer conjugates using bispecific antibodies (BsAbs) and without relying on post-modification chemistry for ligation. Instead, the dual-binding nature of the BsAbs enabled the formation of strong non-covalent interactions, resulting in a highly stable drug carrier.^[475]

Ligand density also plays an important role in determining BBB transport efficacy; and although conclusive design parameters have not yet been agreed upon, the general principles for facilitating optimal brain uptake have been established. An individual ligand’s affinity to its receptor is typically reduced when conjugated to a NP.^[476] However, in vitro and in vivo studies suggest that as the number of conjugated ligands increases (up to a certain point), so too does the NP’s total avidity (strength of binding) to the receptor.^[477-480] Although this is beneficial, at some point the significantly high avidity begins to impede receptor-bound NPs

from undergoing effective transcytosis and being released into the brain compartment.^[477, 478, 480] On the other hand, receptor selectivity has been shown to increase with decreasing affinity of an individual ligand.^[279, 480] Considering such binding behavior, it is suggested that NPs functionalized with an intermediate number of weakly-binding ligands—ligand density should be neither too high nor too low—is likely to facilitate optimal transport across the BBB.^[279, 480] In mice studies using sub-100 nm AuNPs functionalized with Tf, Wiley et al. found that conjugating the NPs with low concentrations of Tf (20–30 molecules per NP) was more effective at achieving BBB transit than conjugating the NPs with high concentrations of Tf (100–200 molecules per NP).^[478] AuNP (20, 45, and 80 nm in size) formulations containing different amounts of conjugated Tf were administered by lateral tail vein injection, and at 8-h post-injection the brains were resected and processed for further analysis. Transmission electron microscopy scanning of the brain sections revealed RMT to be the mechanism by which AuNPs accessed the brain and suggested that this was primarily a TfR-mediated process. Meanwhile, inductively coupled plasma–mass spectroscopy was used to detect and quantify the amount of gold present in the bulk brain, which comprised the blood vessels and parenchyma. When coupled with manual quantitative imaging analysis—a total of 40 tissue section images were visualized using silver enhancement light microscopy, and NPs that had localized in the vessel lumen and parenchyma were separately counted—this approach showed a clear connection between the amount of conjugated Tf and the ability of the NPs to successfully accumulate in the brain parenchyma. Together, these different analytical methods enabled characterization of the transcytosis behavior of Tf-functionalized AuNPs. They revealed that AuNPs with a low ligand density were able to interact effectively with the TfRs and accumulate in the parenchyma to a significant extent, whereas those with a high ligand density remained bound to BMECs and failed to induce effective transcytosis.^[478]

Many of the receptors expressed at the BBB are able to bind and transport multiple

ligands. Therefore, the various RMT mechanisms that are discussed in this section will be grouped according to the specific receptors that can be exploited for BBB transport (see **Table 5** for summary).

Transferrin Receptor (TfR)

TfR, as described previously, is a transmembrane glycoprotein consisting of two 90 kDa subunits linked by a disulfide bridge, each of which can bind one Tf molecule.^[323] It is expressed on hepatocytes, erythrocytes, intestinal cells and rapidly dividing cells (both normal and malignant), as well as the BMECs themselves (this systemic expression is one drawback of targeting the TfR).^[323, 481] There are two different methods of utilizing the TfR pathway to facilitate blood-to-brain transport.

First, endogenous Tf can be covalently coupled to NPs, allowing receptor binding and RMT of the entire drug system.^[192] In terms of ligand density, AuNPs conjugated with 100–200 Tf ligands remain bound to BMECs, whereas AuNPs conjugated with 20–30 Tf ligands have been found to successfully induce transcytosis from luminal to abluminal membrane, before being released into the brain parenchyma.^[478] There is a significant limitation to using Tf, however. Under normal physiological conditions, the amount of endogenous Tf in the plasma is sufficient to almost saturate the available receptors.^[194] Therefore, NPs coated with Tf are forced to compete with circulating Tf for the receptor binding sites, and this reduces the therapeutic concentration that is able to reach the brain.

The alternative is to use mAbs against TfR. mAbs bind to exofacial epitopes on the TfR that do not interfere with endogenous Tf binding sites, thus freeing up NPs to induce RMT without endogenous ligand competition.^[482] Several mAbs have been formulated, including OX26, 8D3, RI7217 and genetically-engineered chimeric mAbs.^[458, 459] In vivo, these mAbs have been found to demonstrate varying levels of targeting efficacy depending upon the species of animal in which they are tested.^[54] For example, OX26, which has been used as an

effective brain drug delivery vector in rats, exhibits suboptimal targeting properties in mice.^[459] Instead, 8D3 and RI7217, both of which are rat-derived mAbs, have been found to demonstrate better targeting to the mouse brain than OX26, achieving brain drug concentrations of 3.1% and 1.6% of the initial dose respectively, compared to negligible brain uptake when OX26 was conjugated.^[459] In studies conducted by Lee et al., the 8D3 antibody was found to achieve the highest murine brain uptake, while the RI7217 antibody showed greater brain selectivity since it was taken up to a lesser extent by the liver.^[459] Just as with endogenous Tf, brain delivery efficiency also depends upon antibody density. For NPs sized 100 nm and carrying up to 10,000 drug molecules per particle, optimal brain delivery was shown for 29 mAbs (OX26) covalently conjugated via a PEG spacer.^[474]

Insulin Receptor (IR)

The IR is a large 300 kDa heterotetramer protein responsible for trafficking insulin through the BBB.^[483] Since insulin, which has a half-life of 10 minutes, is essential for glucose homeostasis, any disturbance of its natural balance can cause hypoglycemia.^[483] As such, even though insulin can be covalently coupled to NPs in an effort to exploit the IR for BBB transport, it is rarely considered a viable option due to safety concerns.^[461] Instead, mAbs against the IR have been engineered, and these have proven effective at inducing RMT without affecting endogenous insulin levels.^[146, 278] Another possible approach involves using insulin-fragments or analogues with retained binding affinity to the IR.

Lipoprotein Receptors

LDLR, LRP1 and LRP2 are closely related, multifunctional, multiligand scavenger and signaling receptors. Whilst mAbs have been successfully engineered against TfR and IR, no antibodies that successfully facilitate RMT have been engineered against the lipoprotein receptors.^[54] Nevertheless, several other ligands, many of which are shared between the lipoprotein receptors, have demonstrated the ability to successfully mediate NP transport

across the BBB. PS 80 is a nonionic surfactant that has proven extremely successful at not only avoiding RES uptake, but also at mediating BBB transport, even though its mechanism of uptake is still not fully understood.^[192] One source suggests that PS 80-coated NPs trigger temporary BBB disruption and thereby gain forced entry into the brain parenchyma.^[484] Most other sources, however, maintain that PS 80 induces adsorption of apolipoproteins such as ApoE or ApoA-I, and that these apolipoproteins in turn interact with lipoprotein receptors on the BBB's luminal surface to trigger RMT of the attached NP drug system.^[192, 371, 382] PS 80-coated NPs have been used to successfully deliver dalargin, kytorphin, loperamide, tubocurarine, doxorubicin and several other drugs to the brain, whilst simultaneously reducing cardiotoxicity and lowering hepatotoxicity.^[192, 474] As an alternative to PS 80, ApoE itself can be directly and covalently ligated to the NP surface, thereby facilitating receptor-mediated uptake followed by transcytosis.^[463, 485, 486] Other members of the apolipoprotein family have also been shown to induce RMT, including ApoB and Apo A-I, the latter of which binds the scavenger receptor class B type I.^[192]

The angiopep family constitutes a set of ligands that are rapidly transported across the BBB via the LRP-mediated pathway. Of these, angiopep-2, which has a molecular weight of 2.4 kDa, is the most promising candidate. When conjugated to the surface of NPs, angiopep-2 facilitates RMT via LRP1, and has been found to display higher transcytosis capacity in vitro and in vivo than other targeting ligands including Tf, lactoferrin (Lf) and avidin.^[341]

Lf and melanotransferrin (MTf) are iron-binding homologs of Tf that may also serve as substrates of the lipoprotein receptors. NPs conjugated with Lf have demonstrated greater brain uptake in rats than those conjugated with either Tf or OX26.^[487] This finding was further validated in a comparative study of the biodistribution properties of Tf- and Lf-conjugated NPs in mice.^[488] Lalani et al. found that PLGA NPs functionalized with Lf achieved 1.62-fold higher brain uptake than those functionalized with Tf, and 3.85-fold higher

uptake than uncoated NPs.^[488] MTF has also been reported as a potentially useful brain-targeting ligand. Although soluble MTF circulates at low blood concentrations (it mainly exists in membrane-bound form), it has been demonstrated to mediate a high rate of transport via LRP1, suggesting its potential as an effective transport vector.^[489, 490] In vivo studies have shown that MTF can achieve 6–8-fold higher mouse brain uptake of the anticancer drug adrimycin than both BSA and Lf.^[491] The MTF–adrimycin drug conjugate has also been found capable of exerting significant tumor-suppressive effects in both rat C6 glioma and human ZR-75-1 mammary tumors.^[491]

RAP is a 39 kDa GTPase whose endogenous function is to assist folding and trafficking of the lipoprotein receptors.^[492] After IV administration, it has been shown to bind the lipoprotein receptors and inhibit clearance of other ligands, including tPA.^[493] Transcytosis of RAP is reported to take place at a faster rate than that of either Tf or MTF.^[464]

Other ligands that are also suggested to bind the lipoprotein receptors include plasminogen activator inhibitor-1 (PAI-1), APP, heparin cofactor II, heat shock protein-96 (HSP-96) and alpha-2-macroglobulin (α 2M).^[194, 278] However, due to the important roles that these molecules play in disease states, as well as their potential adverse effects when delivered therapeutically, they have been investigated to a lesser extent.

Diphtheria Toxin Receptor (DT_R)

The lack of any known endogenous ligands associated with the DT_R makes this receptor an interesting candidate for drug targeting. Exogenous DT could potentially be coupled to NPs to enable RMT, however, the significant enzymatic cytotoxicity imposed by DT renders this approach infeasible.^[494] Instead, cross reacting material 197 (CRM197) is a genetically modified form of DT that has been found to exhibit no toxicity and still mediates transit across the BBB via the DT_R.^[55] A single substitution of glutamic acid for lysine at position 52 eliminates the native toxin whilst maintaining the original receptor-binding affinity.^[466] Since

DT_R is strongly upregulated during pathological conditions, CRM197 could potentially function as a useful targeting vector in a variety of neurological diseases.^[55, 57]

Folate Receptor (FR)

Folic acid has been successfully used as a NP targeting ligand in a number of studies.^[375, 467]

Although expressed on the BBB, the FR is also found on a few other tissues including the choroid plexus, thyroid, and kidneys.^[495] Additionally, it can act as a tumor targeting ligand due to its overexpression on various tumoral cells.^[474] PEG-PLGA micelles loaded with doxorubicin and functionalized with folic acid were found to accumulate not only in the brain of mice, but specifically in the tumor tissues to a significant extent.^[496] Paclitaxel-loaded PCL-MPEG micelles also functionalized with folic acid were shown to exhibit more potent effects on cancer cells in the brain than non-targeted micelles.^[324]

FC5 Orphan Receptor (TMEM30A)

The FC5 sdAb has been shown to initiate clathrin-mediated endocytosis followed by complete transcytosis across the BBB (without intracellular trafficking to the lysosomes).^[278] Although most studies to date have focused on the modular incorporation of FC5 sdAbs into fusion antibodies and antibody–drug conjugates, it is no stretch to assume that functionalization of NPs with FC5 sdAbs offers a potentially promising approach to CNS drug delivery.^[469]

Functionalization with Cationic/Amphiphilic Constituents for AMT: NPs typically require functionalization with a positive surface charge to facilitate AMT across the BBB. This can be achieved by building inherently cationic or amphiphilic NPs, or by functionalizing the NP surface with positively charged ligands including CPPs (see Table 5 for summary).^[196]

Cationic/Amphiphilic NPs

NPs that exhibit cationic surface charge at pH 7.4 can be achieved through assembly from positively charged components. As previously discussed, some of the most commonly used building blocks are composed of DOTAP mixed with DOPE, but other polymers and block

copolymers are also commonly used.^[199, 417] One potential drawback of cationic NPs is that they may exhibit toxicity in certain applications compared with both anionic and neutral NPs.^[396] Amphiphilic NPs, however, have been found to be effective at prolonging circulation time and improving brain drug delivery in vivo without causing significant toxic effects (**Figure 38**).^[449, 497, 498] In both rat and murine models, Calvo et al. observed that amphiphilic NPs composed of PEG-poly(cyanoacrylate) block copolymer were able to circulate in the blood longer (after intravenous administration) and penetrate the brain to a greater extent than non-amphiphilic NPs, even those coated with PS 80 and poloxamine 908.^[449, 497] This difference was found to be even more striking during pathological situations where BBB integrity was likely to be compromised.^[449, 497]

Cationic Ligands

CPPs are short peptides, usually less than 30 amino acids in length, that serve as cationic ligands for the AMT system. Although some CPPs contain only positively charged residues to facilitate efficient cellular internalization, others have an amphipathic surface structure—alternating hydrophobic and hydrophilic residues—to enable stealthy interactions with cell membranes (**Figure 39**).^[188] The same reasoning as applied earlier would suggest that amphipathic CPPs are more effective than purely cationic ones.

Many CPP sequences have been discovered and investigated, including model amphipathic peptide (MAP), Antennapedia (Antp), transportan, penetratin, fusion sequence-based peptide (FBP), transactivator of transcription (TAT), and the protegrin-derived pegelin proteins (e.g., Syn-B1, and Syn-B3).^[10, 501-503] These CPPs display varying levels of transfection efficacy and cytotoxicity. TAT, for example, when conjugated with various peptide compounds, has been found non-toxic towards rat cells in vitro at concentrations less than 10 μM ,^[504] and when conjugated with ritonavir-loaded NPs demonstrates an 800-fold increase in mouse brain drug concentration compared to free drug at two weeks (**Figure**

40).^[470] Antp, on the other hand, has been found significantly more toxic than TAT when administered at concentrations of up to 10 μM .^[504] In general, the common tendency of CPPs is to exhibit chain length-dependent and dose-dependent toxicity; a significant increase in either can potentially lead to increased BBB permeability, but also to BMEC apoptosis and neuroinflammation.^[197, 504] Another potential drawback of CPPs is their tendency to induce nonspecific entry into various cells, including those in peripheral tissues.^[196] In terms of benefits, however, CPPs have been known to transport both small and large drug systems through the BBB, and in much greater amounts than either free drugs or uncoated NPs.^[196, 470]

Other cationic ligands have also been shown to facilitate AMT. TMC is a permanently quaternized chitosan derivative that is positively charged under physiological conditions. When conjugated to the surface of pre-formed PLGA NPs via a carbodiimide link, TMC was shown to facilitate drug distribution to the cortex, paracoele, third ventricle and choroid plexus of the mouse brain, while uncoated PLGA NPs failed to penetrate the BBB at all.^[195] This was achieved without inducing toxicity, thus proving TMC's potential as a targeting moiety. In a separate experiment, AuNPs coated, using layer-by-layer assembly,^[306, 505] with polyelectrolytes (PSS/PAH) and HSA were able to circulate for long periods, evade immune system recognition and induce AMT through the BBB.^[395] Compared to control AuNPs functionalized only with PAH/PSS, the AuNPs coated with PAH/PSS and HSA demonstrated lower cytotoxicity at the same concentrations.^[395] Polyamine modification of other conjugated proteins (insulin, IgG, etc.) has also proven a viable method of inducing AMT, although the toxic and immunogenic consequences have yet to be fully dealt with.^[506]

NP Functionalization to Encourage Immune Cell Internalization, Trafficking and Release ("Trojan Horse" Strategy): The design rules that need to be applied to NPs to facilitate cell-mediated transport are different to the aforementioned targeting methods. Instead of being functionalized to avoid RES uptake, drug-loaded NPs are instead willfully designed to be

taken up by the immune cells, before being trafficked into the brain and released under inflammatory stimulation.^[199] In vitro and in vivo studies have shown that NP formulations, especially those with magnetic targeting capabilities, may be better suited to cell-mediated delivery than free drugs due to their potency and specificity.^[200, 201, 507, 508]

Several factors must be taken into consideration when designing nanomaterials for cell-mediated transport, including immune cell loading capacity, intracellular preservation, release rate and toxicity. After being opsonized, NPs are recognized and bound by cell-surface receptors, including the mannose, complement and Fc receptors (MR, CR and FcR).^[199] It has been shown that both positively and negatively charged NPs can accumulate in immune cells (specifically mononuclear phagocytes) to a greater extent than neutral NPs.^[199] However, there is still an ongoing debate as to whether cationic NPs or anionic NPs are more readily internalized (evidence suggests that anionic NPs are sequestered at a faster rate than cationic NPs).^[199, 319-321] Shape also appears to play an important role in determining whether or not phagocytosis occurs. Studies conducted by Champion et al. suggest that whilst particle size impacts upon the completion of phagocytosis, particle geometry determines whether phagocytosis occurs in the first place, or whether macrophages simply spread out over the particles instead.^[509] For polystyrene particles, those with very high aspect ratios (>20) and low curvature (e.g., worm-like shape) were found to inhibit phagocytosis more successfully than those possessing high curvature geometries (e.g. elliptical or spherical shape).^[510] This was elsewhere confirmed by separate studies using polystyrene particles and filomicelles (highly stable, polymer micelle assemblies).^[511, 512] However, in a contradictory study published by Gratton et al., hydrogel particles with rod-like, high-aspect-ratio geometries were found to be internalized faster and more efficiently than symmetrical, low-aspect-ratio particles.^[513] Thus, it can be concluded that cellular internalization efficiency is not solely dependent upon particle geometry, but also upon a whole host of other, interacting factors,

such as flow-based effects,^[514, 515] rigidity,^[516, 517] and surface charge and chemistry of the NPs.^[518-520]

Immune cells possess innate lysosomal clearance functions that can lead to enzymatic degradation of the internalized NPs. Studies have shown that positive surface charge can prevent NPs from being trafficked to the lysosome and enzymatically degraded. Meanwhile, in some studies, neutral NPs have been shown more susceptible to lysosomal destruction, and negative NPs had even greater levels of degradation.^[199]

NP release from immune cell carriers is an area that is still under active investigation. It has been shown that elevated intracellular Ca^{2+} levels and mild hypothermia both facilitate the controlled release of drug-loaded NPs from immune cells.^[199] It is also known that anionic NPs have effective release profiles, while cationic NPs demonstrate poor release kinetics.^[199]

Ultimately, it is unlikely that a single NP formulation can ensure optimal performance in all these areas (e.g., loading capacity, intracellular preservation, release kinetics and toxicity), and therefore a tradeoff must be made to ensure that cell-mediated delivery is efficacious. In a study conducted by Zhao et al., different NP formulations incorporating the enzyme catalase were tested to determine their suitability for cell-mediated transport.^[521] Anionic NPs (built from block copolymers) were found to exhibit low cytotoxicity, high immune cell loading capacity and effective release rates, but they lacked protection against enzymatic degradation. On the other hand, cationic NPs (also built from block copolymers) were found to preserve catalase efficiently, but demonstrated cytotoxicity and low immune cell loading and release kinetics. Finally, NPs encased in a PEG corona showed water stability, limited cytotoxicity and efficient intracellular protection, but at the same time displayed diminished loading capacity and release rates.^[521] Based on a comparative analysis of these properties, it has been tentatively suggested that the optimal candidate for cell-mediated drug delivery is the NP formulation based on cationic block copolymers (PEI-PEG

or PL-PEG).^[199, 521] To further increase uptake into immune cells, these types of NP formulations can be functionalized with mannosamine, a ligand known to increase internalization via the mannose receptor.^[199, 522] Another ligand is the Arginine-Glycine-Aspartate (RGD) peptide, which has been observed to bind to integrin receptors expressed on polymorphonuclear immune cells and stimulate phagocytosis (**Figure 41**).^[523]

Cell-mediated transport can be combined with magnetic targeting to further enhance brain drug delivery. NPs embedded with a magnetic core are taken up by immune cells and then actively targeted to the brain under the guidance of an external magnetic field. By augmenting the natural BBB targeting process in this way, site-specific drug uptake can be increased and off-target effects can be reduced.^[200] In rat studies, Jain et al. found that negatively charged magnetic liposomes anchored with RGD peptide achieved brain drug levels ~9-fold higher than free drug solution, ~6-fold higher than non-magnetic liposomes, and 1.5-fold higher than uncoated magnetic liposomes.^[200] In addition, liver uptake decreased by ~48% (down from ~51% to ~27% of the initial dose) for the RGD-coated liposomes when a magnetic field was applied. Collectively, these results suggest that magnetic targeting has the potential to enhance the efficacy of cell-mediated brain targeting while simultaneously maintaining optimal systemic biodistribution properties.

6. Inside the CNS: Targeting Strategies and Clearance Mechanisms

A suitable drug (delivery) system, whether modified drug or nanoparticulate carrier, should essentially have two targeting functionalities—one to cross the BBB, and another to reach and penetrate the pathologically affected target region.^[40] This Review has focused, in large part, on the former, but it is important to take into consideration possible methods of targeting *within* the brain parenchyma. Drugs that are transported through the brain only via simple diffusion may face a number of significant challenges including potential cellular

obstructions, entrapment in dead-space microdomains, viscous drag imposed by ECM macromolecules, drag arising from the channel walls, and transient, non-specific binding to cell membranes or the ECM.^[11] These factors represent major challenges to deriving any significant therapeutic benefit from a drug formulation, and therefore parenchymal targeting methods need to be devised. Some of these methods are briefly outlined in Section 6.1 and 6.2. In addition, the various mechanisms of CNS solute clearance should also be considered, as these are likely to be of relevance in the context of drug efficacy and safety. Although relatively few studies have been conducted in this area,^[524] four potential CNS clearance mechanisms will be discussed in Section 6.3.

6.1. Passive Blood Circulation and Extravasation

When a drug is administered systemically (e.g., intravenously), targeting within the body can be separated into (i) *passive blood circulation and extravasation* and (ii) *active targeting*.

Passive blood circulation and extravasation is a process directly dependent upon drug survival time, and refers to the accumulation of drugs in specific tissues as a result of distinct biological conditions.^[525] The brain itself is well-perfused, comprising approximately 100 billion capillaries with a combined length of almost 400 miles.^[526] Under normal conditions these capillaries are estimated to be several micrometers in diameter, although significant constriction can occur as a result of noradrenergic innervation.^[527] In general, such dimensions permit easy passage of drugs through the cerebral vasculature.^[4] The greatest challenge to CNS drug delivery is instead posed by the BBB; collectively, its physical, enzymatic, transport and immunological defenses render the passive accumulation of drugs in the CNS almost impossible.^[4] However, the enhanced permeability and retention (EPR) effect, which may occur in certain disease states, is believed to play an important role in enabling passive (or active) drug delivery to some brain tumors or inflamed CNS tissues

(Figure 42).^[280, 528, 529] During tumor growth or inflammation, the rapid proliferation of cells puts an increased demand on the supply of oxygen and nutrients. Existing blood vessels are not enough to meet this demand, and angiogenesis is therefore stimulated by VEGF and other growth factors.^[531] However, the rapid rate of tumoral growth causes new blood vessels to form in an aberrant and disordered fashion.^[531] Tumoral vascular endothelial cells show poor alignment, resulting in wide fenestrations, lack of smooth muscle, impaired functional receptors and compromised lymphatic drainage.^[532] The end result is that the tissues can become “leaky” and poorly perfused, and this phenomenon is referred to as the EPR effect.^[532-534] While the EPR effect is disadvantageous in that it may contribute to the proliferation of tumor tissues, it is proposed to be beneficial for drug delivery as it may lead to higher local drug concentrations.^[47] NPs can extravasate into the tumor interstitium through the hyperpermeable vasculature and then remain in diseased tissues for extended periods due to decreased levels of lymphatic drainage.^[407] Although much attention has been drawn to the EPR effect in the context of non-CNS tumors (although it still remains controversial)^[530], separate animal studies have shown that it can also be exploited by NPs in various neurological conditions, including gliomas,^[377] ischemia,^[535] and other diseases involving neuroinflammation.^[536] Additionally, the lack of oxygen and nutrient supply caused by the EPR effect induces glycolysis to occur in tumor tissues as a compensation mechanism, thereby creating an acidic local environment.^[351, 537] In an effort to capitalize on this, NPs can be designed to maintain their stability in a neutral pH environment but then release their payload in an acidic environment (this is an active targeting technique).^[537]

However, despite this rationale for the development of passive targeting strategies, recent studies suggest that the EPR-model is not always effective and reliable for drug delivery.^[530, 538] Nichols and Bae posit that EPR-dependent drug delivery is compromised by a number of factors including high tumor interstitial fluid pressure (IFP), which hinders the

ability of NPs to extravasate into the tumor, highly irregular and chaotic tumor vasculature, and poor blood flow.^[538] They also suggest that the EPR effect observed in animal models is not sufficiently representative of that in human tumors, and thus constitutes a reason why several drug formulations have failed in clinical trials (**Figure 43**).^[538] In an effort to curb these failures, it has therefore been proposed that therapeutic nanomedicines should be studied on more clinically relevant tumor models, and should only be engineered against the EPR effect only on a case-by-case basis.^[530, 538]

6.2. Active Targeting

6.2.1. Exploiting Environmental Conditions of Disease Sites

Active targeting of NPs can enhance the effects of passive circulation and extravasation by providing greater disease specificity. There are several ways this can be accomplished (**Figure 44**). One method that has already seen effective widespread use is magnetic targeting. Although MNPs hold significant potential for drug delivery to the brain, they also offer benefits for drug delivery within the brain, extending beyond the EPR effect.^[407] After crossing the BBB, superparamagnetic NPs can be subjected to an external magnetic field and guided to diseased regions where they can then exert their therapeutic effects. A number of studies have demonstrated improved drug accumulation and retention as a result of this approach.^[407, 426] In the case of glioma, magnetically targeted NPs were found in 11.5-fold higher concentrations in rat tumor tissues than were non-magnetic NPs.^[407] Preclinical and clinical trials have shown that MNPs are fairly non-toxic and biocompatible with human CNS tissues after IV administration.^[408, 409]

In most neurodegenerative disorders, the disruption of brain physiology creates an abnormal local environment that can be harnessed for drug delivery purposes. NPs can be functionalized to respond to specific cellular conditions associated with CNS pathology,

including low pH, temperature change and hypoxia. In the case of pH, acidic environments created by inflammatory and tumor tissues can be exploited by incorporating pH-responsive components into the drug system (Figure 44A). For example nitric oxide (NO), which plays a role in regulating multiple cellular processes associated with pathology, can be covalently linked with polyamine-stabilized AuNPs.^[539] When the resulting drug system is trafficked into mildly acidic environments (~pH 6.8 inside inflammatory and tumor tissues, pH 5.5–6 in endosomes, pH 4.5–5 in lysosomes),^[539, 540] NO is released to act therapeutically on the target region.^[539] In a separate study, Du et al. designed a dual pH-sensitive polymer NP for the efficient delivery of doxorubicin into breast cancer stem cells (**Figure 45**).^[541] Their nanoparticulate system was capable of reversing surface charge from negative to positive in the vicinity of tumor tissue (~pH 6.8), with the purpose of facilitating enhanced cellular internalization. After endocytosis, the further decreased pH of the intracellular compartments triggered site-specific doxorubicin release, ultimately resulting in improved cytotoxicity towards the tumor cells.^[541] Reversal of surface charge was achieved by acid-responsive cleavage of an amide bond (between the amino groups of the polymer and 2,3-dimethylmaleic anhydride (DMMA)), while the release of doxorubicin was achieved by cleavage of an acid-labile hydrazone bond (between doxorubicin and the polymer). At pH 5.0, more than 75% of the initial doxorubicin dose was cumulatively released by 184 hours, whereas at pH 6.8 approximately 25% was released by 184 hours.^[541] Thus, the ability of this NP to respond to both extracellular and intracellular triggers reveals its suitability for targeted drug delivery to tumors. Although the aforementioned proof-of-concept formulation was tested specifically on breast cancer cells, the underlying technology could be feasibly translated to the CNS, where similar intracellular and extracellular pH changes have been observed as a result of malignant gliomas,^[542] AD,^[543] cerebral ischemia,^[544] and even depression.^[544] Potential causes for these

changes include pathological environmental conditions (e.g. hypoxia;^[542] presence of metals)^[543] and aberrant gene expression.^[545]

In a similar manner, abnormal temperature changes associated with some types of CNS pathologies can be exploited by building thermally-responsive linkers into the NP structure (Figure 44B).^[539] When these linkers—made up of nucleic acids, peptides, lipids, carbohydrates, polymers, etc.—are exposed to a characteristic temperature or temperature range, they become disrupted and subsequently trigger the release of the drug molecules. The “trigger” temperature, as it is so-called, can be chemically adjusted to suit different biological requirements.^[539] For example, Pradhan et al. formulated temperature-sensitive folate-targeted doxorubicin-containing magnetic liposomes (MagFolDox liposomes) for the thermo-chemotherapy of cancer.^[546] In KB and HeLa cell lines, these liposomes, which co-encapsulated both magnetic NPs and doxorubicin, were able to be magnetically guided to the target region before initiating folate receptor-mediated uptake (via folate ligands on the liposomal surface). Once in the target region, the generation of heat via an AC magnetic field resulted in the temperature-sensitive release of doxorubicin (**Figure 46**).^[546] This temperature-sensitivity property was achieved by incorporating 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol) (DSPE-PEG) into the liposome’s structure, the combination of which showed a transition from gel to liquid crystalline phase at 43°C. The phase change was responsible for increased leakiness of the lipid bilayer, resulting in drug release. In comparison to non-magnetic folate-targeted liposomes and Caelyx (a clinically approved liposomal formulation of doxorubicin), these MagFolDox liposomes were found to elicit the greatest anti-tumoral effects—albeit in *in vitro* KB (human epidermoid carcinoma) and HeLa (human cervical carcinoma) cancer cells—when synergistically combined with magnetic hyperthermia.^[546] In a separate study of glioma-bearing rats, Jiang et al. administered pH- and

temperature-sensitive magnetic nanogels conjugated with dye-labeled lactoferrin (Cy5.5-Lf-MPNA nanogels) via direct brain injection, and observed that they were able to respond with high sensitivity to the altered temperature and pH of the glioma environment.^[547] Although the current literature surrounding temperature-sensitive CNS therapeutic development is fairly sparse, potential disease applications for the aforementioned technology include cerebral ischemia,^[548, 549] traumatic brain injury,^[550] and gliomas.^[547]

In addition, redox-triggered drug systems have been shown to enable site-specific drug release without perturbing homeostasis (a potential drawback of the other approaches).^[551] By functionalizing doxorubicin-loaded liposomes with ferrocene-modified phospholipids, Noyhouzer et al. were able to synthesize a smart drug delivery system based on redox functionality. The ferrocene-based molecules served as a redox trigger, causing drug release in response to local electrochemical changes in the vicinity of tumor tissues (**Figure 47**).^[551] Although this study was conducted using adenocarcinoma HeLa cervical cancer cells, it has been shown elsewhere that significant changes in redox state can occur as a result of neurological injury. AD,^[552] hypoxic ischemia^[553] and seizure^[554] have been shown to induce significant redox changes in CNS tissues (specifically, in the hippocampus for AD and seizure patients,^[552, 553] and in mitochondria near the occluded vessel for ischemia)^[554] as a result of oxidative stress, suggesting a potential opportunity for the development of redox-triggered therapeutics (Figure 44C).

6.2.2. Functionalization with Targeting Ligands

While the aforementioned drug systems (except for magnetic targeting) can be triggered by environmental stimuli, another active targeting approach involves ligating free drug molecules or NPs with disease-specific targeting ligands (Figure 44D). Note that these are different from the ligands used to cross the BBB. They aid drug systems in localizing in target regions and exerting therapeutic effects, but they play no role in actually facilitating passage through the

BBB. Functionalization of NPs with these two different ligands can confer upon them “cascade” dual-targeting characteristics—the ability to cross the BBB as well as the ability to actively penetrate diseased regions in the brain. For example, in an AD mouse model study, Zhang et al. functionalized PEGylated PLA NPs with two targeting ligands, TGN and QSH. TGN (or TGNYKALHPHNG), a 12-amino acid sequence ligand, was selected for its proven ability to induce BBB crossing (3.6 times more accumulation in the brain than unmodified NPs). QSH (or QSHYRHISPAQV), a D-enantiomeric peptide, was selected as the second-order ligand due to its ability to successfully bind $A\beta_{1-42}$ (the main component of amyloid plaque) without eliciting immunogenic effects or succumbing to protease degradation. After injection into the tail vein, the dually-functionalized NPs were found to successfully enter the brain and bind with high affinity to the $A\beta_{1-42}$ plaques.^[555] Although this study confirms the rationale behind dual-targeting, it still remains to be seen whether this strategy is actually efficacious for the clinical treatment of AD.

In a few other cases of dual-targeting, the same ligands that elicited BBB penetration were also able to penetrate diseased regions.^[556-558] Using in vitro models of brain glioma, Li et al. showed that pH-sensitive PAMAM dendrimers functionalized with Tf on the exterior and tamoxifen (TAM) in the interior were capable of achieving dual-targeting, both across the BBB and into glioma cells.^[556] The mechanism of action at the BBB involved RMT via interaction of Tf with the TfR, simultaneously coupled with TAM’s inhibition of the luminal efflux proteins. Similar protein expression patterns were observable on C6 glioma cells, thereby allowing the same mechanism of action to facilitate drug penetration of the tumor-affected regions.^[556] Results showed that the dual-targeting carrier was able to deliver doxorubicin more successfully to glioma cells (i.e. more optimal release kinetics) compared to carriers lacking the dual-targeting characteristic.^[556]

Since changes in neurological anatomy and physiology are not uniform for all diseases, active targeting ligands generally require individual tailoring to suit the requirements of each condition.^[11] Several such ligands have been discovered and are presently being investigated for their clinical implications.^[559] Chlorotoxin (CTX), for example, has been selected as a tumor-targeting ligand due to its strong affinity for tumors of neuroectodermal origin.^[560] In studies conducted by Veisoh et al., iron oxide NPs coated with PEGylated chitosan-branched copolymer (for stability) and CTX were able to selectively bind to glioma, medulloblastoma and sarcoma cells without accumulating in nonneoplastic tissues to any great extent.^[560] The RGD peptide, as well as the similar internalizing RGD peptide (iRGD), have been separately identified as ligands possessing strong affinity and selectivity for tumor tissues.^[410, 561, 562] NPs conjugated with RGD and iRGD sequences have been shown to accumulate in glioma cells as a result of selective binding to $\alpha\beta3$ integrin receptors overexpressed on their surface.^[410, 561] Although several such active targeting ligands have been explored in isolation, many of these remain to be fully evaluated in the context of a dual-targeting approach.

To date, antibodies (mAbs and sdAbs) have been mostly used in a standalone therapeutic capacity (some of these are discussed below). Nevertheless, in vivo studies have confirmed that they can also be conjugated to NPs and feasibly used as active targeting vectors in the brain,^[563] although results have been conflicting thus far. In malignant tumors, for example, it is still unclear what role active targeting ligands play in contributing to tumor localization and uptake.^[564-567] Nevertheless, several therapeutic mAbs have been engineered against disease targets in AD, MS, PD and West Nile Virus (WNV) encephalitis.^[54] In the case of AD, therapeutic mAbs have been developed against various targets (**Figure 48**), including the amino terminus of the human A β peptide, against β -secretase 1 (BACE1),^[279, 568] and against metabotropic glutamate receptor 1 (mGluR1).^[54] All three of these targets—A β , BACE1 and mGluR1—play important roles in perpetuating the pathological processes

involved in AD. For MS, a therapeutic mAb has been engineered against the leucine-rich repeat and Ig-containing Nogo receptor interacting protein-1 (LINGO-1). Meanwhile, one of the hallmarks of PD is the accumulation of deposits in the brain caused by abnormal processing of the protein synuclein-1. mAbs against synuclein-1 have therefore been proposed as treatments for PD.^[54] Other studies have shown that neurotrophic growth factors expressed in the CNS—including BDNF—have the potential to act as protective agents against brain ischemia or injury.^[570] By conjugating BDNF to the OX26 mAb via avidin-biotin technology, Zhang and Pardridge were able to formulate a bi-functional therapeutic that possessed the ability to cross the BBB via the interaction of OX26 with TfR, and subsequently act on neurons via binding of BDNF to the neuronal trkB receptor.^[571] By combining BBB targeting functionality with active neuron targeting functionality, they successfully demonstrated that neuroprotective drugs such as BDNF could be delivered intravenously rather than via neurosurgical interventions.^[571] Although the aforementioned antibodies have been engineered for incorporation in recombinant fusion proteins, it is surmised that they have potential to be used as targeting or therapeutic moieties in NP formulations as well.

6.3. Clearance from the Brain

After a drug has successfully acted on its intended target region, the final consideration that needs to be made is its clearance from the CNS. As previously discussed, the ISF and CSF volumes experience frequent turnover, and therefore most drugs are cleared rapidly from the parenchyma.^[24] As a result, the issue that needs to be addressed is usually not drug clearance from the CNS, but drug retention within the CNS. Regardless, mechanisms of drug clearance are closely related to the efficacy and safety of brain drug delivery, and yet very few studies have been conducted in this area.^[524] Toxicity is a key consideration for nanomaterial-based CNS therapeutic strategies and has therefore been explored in a number of studies.^{[270, 345, 388,}

393, 395, 399, 402, 504, 524] There are several factors that can impact upon a drug's toxicological profile, and drug clearance is an important aspect that needs to be considered.^[524] Jiang and Gao postulate that there may be four mechanisms of NP clearance from the brain: (i) extracellular degradation by metabolizing enzymes, (ii) internalization and subsequent degradation by cells of the NVU, (iii) entrance into CSF bulk flow and reabsorption into the bloodstream or cervical lymphatics, and (iv) brain-to-blood efflux via abluminal transporters.^[524] It has already been established that several families of enzymes (including monoamine oxidases, CYP450s, epoxide hydrolases and hyaluronidases, among many others) exist at the site of the BBB and within the brain parenchyma, and it is likely that these could contribute to extracellular therapeutic breakdown.^[524, 572] In addition, studies suggest that NPs can be taken up by cells of the NVU, including neurons and astrocytes.^[573, 574] It is hypothesized that these cell types could trigger specific intracellular pathways leading to degradation of the internalized drug systems.^[524] In a study conducted by Maysinger et al., live transgenic mice were imaged in real-time to determine their CNS responses to subcutaneously injected quantum dots (QDs).^[573] Results showed that the administration of PEGylated QDs caused transient astrocyte activation, culminating in the localization of these QDs in the lysosomes of astrocytes within one week.^[573] This appears to confirm the suggestion that therapeutic breakdown could occur within the cells of the NVU. The other two mechanisms of drug elimination from the CNS (CSF drainage and brain-to-blood efflux) have already been discussed to some extent in Sections 2.1 and 2.4.

Regardless of these findings, therapeutics can still be engineered to engage in bidirectional transport across the BBB. Due to the polarized nature of BMECs, transporters and receptors are not expressed uniformly at luminal and abluminal membranes. Instead, some transporters and receptors only exist at the abluminal membrane, facilitating the movement of substrate molecules solely in the direction of brain-to-blood. These include the

neonatal Fc receptor (FcRn), which is responsible for the selective efflux of IgG molecules from the CNS,^[54] and various amino acid transporters and other peptide transporters.^[21] By functionalizing drug systems with moieties that bind to these abluminal transporters/receptors, they can be made to traverse the BBB in the brain-to-blood direction. The FcRn is especially significant, since it has been exploited by BsAbs to facilitate drug clearance from the CNS.^[146]

BsAbs constitute a sub-class of recombinant fusion proteins that are created by merging two antibody fragments together via linker or “spacer” peptides.^[575] This fusion creates a complete drug system comprising triple functionality: (i) a targeting Ab at the “head” of the molecule that mediates RMT of the drug from blood to brain; (ii) a therapeutic Ab at the “tail” end of the molecule that binds to disease targets within the parenchyma and exerts its remedial effect; and (iii) a targeting “midsection” domain that mediates efflux via reverse transcytosis across the BBB.^[146] These three functionalities have all been explored to some extent in this Review. The targeting mAb can be engineered against various receptor targets including the TfR, HIR or FC5 orphan receptor. The therapeutic mAb can be engineered against specific disease targets in AD, MS, PD, WNV encephalitis or other conditions of neurological origin. The “midsection” domain can be engineered with a CH2-CH3 interface that serves as the binding site for the BBB FcRn.^[54] Combined, these three domains facilitate bidirectional transport across the BBB, as well as specific targeting to diseased regions of the brain.

7. Conclusions and Future Directions

It is becoming increasingly evident that new therapeutics and medical interventions are necessary if we are to find radical solutions to the challenges posed by neurological diseases. Most current drug therapies have proven ineffective at curing or even curbing the progression

of many pathologies of the CNS, and in large part this has been due to the role played by the BBB. It does not help that the exact disease mechanisms underlying most of the major neurological conditions remain unidentified. Going forward, nanomedicine holds promise for the development of successful strategies to penetrate the BBB and treat specific disease targets. The combination of nanoscale drug (carrier) engineering with improved understanding of disease etiology is fundamental for deriving novel therapeutic approaches. In this regard, therapeutic agents can be formulated as free drugs, and they can also be associated with NPs via adsorption, dissolution, encapsulation or covalent bonding.^[192]

Despite the promise offered by precisely engineered free drugs (e.g., recombinant fusion proteins possess multiple functionalities enabling BBB transit, disease treatment and subsequent brain efflux),^[54] NPs offer unique advantages that may make them suitable for brain drug delivery. This Review has explored in some depth different types of NPs—including lipid-based NPs, polymeric NPs and inorganic NPs—as well as the different techniques by which they can be modified for successful targeting to (and within) the brain. In conclusion, it is this formulation and functionalization of NPs that can render them so useful for clinical applications. A desirable NP formulation could comprise some or all of the following functionalities:

- A core with magnetic and/or optical properties (e.g. MNPs, AuNPs) to facilitate: (i) localization of the particle at the target site by remote actuation, (ii) imaging of the particle's location in the body, and (iii) release of the therapeutic agent(s) by remote actuation.
- A 'smart' polymeric outer coating that possesses precisely tunable properties (e.g. a degree of hydrophobicity for drug encapsulation; configuration of functional groups for reversible drug attachment and surface modification) and that can be easily endowed with added functionality to ensure adequate NP biocompatibility and circulation time

without recognition by the RES (unless utilizing cell-mediated transport).

- One or more therapeutic cargos (molecule or biologic) that can be encapsulated or bound to the NP and that can act specifically via the desired mechanism of action.
- A luminal BBB targeting ligand, to initiate transport in the blood-to-brain direction.
- A parenchymal targeting ligand that binds selectively to the disease target.
- A linker molecule that can be triggered to release the therapeutic agent(s) at the target site, based on environmental stimuli (e.g., pH, temperature, redox) or external stimuli (e.g., optical/magnetic stimulation).
- An abluminal BBB targeting ligand, to mediate brain-to-blood efflux of the NP (this functionality is not always necessary, since various endogenous mechanisms already contribute to the elimination of drugs from the CNS).

Although significant progress has been made thus far, we are not yet at the stage where NPs can be readily used for the clinical treatment of neurological diseases. One of the most pressing issues that needs to be addressed is finding effective, reliable and non-toxic methods for crossing or bypassing the BBB.^[8] To this end, minimally invasive or noninvasive methods should be explored to a greater extent. Intranasal administration, for example, is a patient-friendly administration route, and could be a feasible approach if brain drug concentrations via this route are enhanced.

Another area of focus should be the selectivity of drug targeting. Many of the current NP formulations deliver drug agents relatively indiscriminately to various parts of the body, resulting in potential systemic toxicity. Yet the distribution of transporters and receptors on the BBB is heterogeneous in nature.^[28] If selective transporters or receptors on the BBB can be targeted, and that too in disease-affected regions of the brain (e.g., the IL-1 transporter is highly concentrated at the posterior division of the septum,^[576] while TNF is primarily transported into the hypothalamus and occipital cortex),^[28] the therapeutic effects will be

narrowed to the region of interest rather than scattered elsewhere. Finally, drug agents should be studied to improve their pharmacokinetic properties within the brain compartment itself. The slow rate of diffusion within the parenchyma tends to inhibit CNS drug distribution, and this warrants a more effective strategy. Externally guided magnetic targeting^[407] or even remotely-actuated nanodevices^[577] may provide an effective means of facilitating drug movement to, and within, the brain. Indeed, nanomedicine is a burgeoning field with great potential, but much work is still to be done if the technologies currently under investigation are to eventually make the transition to clinical practice.

Author information

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References

- [1] P. Ehrlich, *Das Sauerstoff-Bedürfniss des Organismus: eine farbenanalytische Studie*, Hirschwald, Berlin, Germany **1885**.
- [2] N. J. Abbott, L. Ronnback, E. Hansson, *Nat. Rev. Neurosci.* **2006**, *7*, 41.
- [3] B. V. Zlokovic, *Nat. Rev. Neurosci.* **2011**, *12*, 723.
- [4] W. M. Pardridge, *Mol. Interventions* **2003**, *3*, 90.
- [5] N. J. Abbott, *J. Inherited Metab. Dis.* **2013**, *36*, 437.
- [6] K. E. Schlageter, P. Molnar, G. D. Lapin, D. R. Groothuis, *Microvasc. Res.* **1999**, *58*, 312.
- [7] S. Krol, R. Macrez, F. Docagne, G. Defer, S. Laurent, M. Rahman, M. J. Hajipour, P. G. Kehoe, M. Mahmoudi, *Chem. Rev.* **2013**, *113*, 1877.
- [8] W. M. Pardridge, *Drug Discovery Today* **2007**, *12*, 54.
- [9] X. Yi, D. S. Manickam, A. Brynskikh, A. V. Kabanov, *J. Controlled Release* **2014**, *190*, 637.
- [10] Y. Chen, L. Liu, *Adv. Drug Delivery Rev.* **2012**, *64*, 640.
- [11] M. Masserini, *ISRN Biochem.* **2013**, *2013*, 18.
- [12] M. Björnmalm, K. J. Thurecht, M. Michael, A. M. Scott, F. Caruso, *ACS Nano* **2017**, *11*, 9594.
- [13] T.-T. Zhang, W. Li, G. Meng, P. Wang, W. Liao, *Biomater. Sci.* **2016**, *4*, 219.
- [14] M. F. Bennewitz, W. M. Saltzman, *Neurotherapeutics* **2009**, *6*, 323.
- [15] J.-C. Olivier, *NeuroRx* **2005**, *2*, 108.
- [16] S. Parveen, R. Misra, S. K. Sahoo, *Nanomed. Nanotechnol. Biol. Med.* **2012**, *8*, 147.
- [17] P. Mulvaney, W. J. Parak, F. Caruso, P. S. Weiss, *ACS Nano* **2016**, *10*, 9763.
- [18] M. Björnmalm, M. Faria, F. Caruso, *J. Am. Chem. Soc.* **2016**, *138*, 13449.
- [19] P. Ballabh, A. Braun, M. Nedergaard, *Neurobiol. Dis.* **2004**, *16*, 1.
- [20] N. J. Abbott, A. A. K. Patabendige, D. E. M. Dolman, S. R. Yusof, D. J. Begley, *Neurobiol. Dis.* **2010**, *37*, 13.
- [21] B. V. Zlokovic, *Neuron* **2008**, *57*, 178.
- [22] B. T. Hawkins, T. P. Davis, *Pharmacol. Rev.* **2005**, *57*, 173.
- [23] N. J. Abbott, *Neurochem. Int.* **2004**, *45*, 545.
- [24] W. M. Pardridge, *Fluids Barriers CNS* **2011**, *8*, 7.
- [25] B. Engelhardt, P. Vajkoczy, R. O. Weller, *Nat. Immunol.* **2017**, *18*, 123.
- [26] J. Lok, P. Gupta, S. Guo, W. J. Kim, M. J. Whalen, K. van Leyen, E. H. Lo, *Neurochem. Res.* **2007**, *32*, 2032.
- [27] E. A. Neuwelt, B. Bauer, C. Fahlke, G. Fricker, C. Iadecola, D. Janigro, L. Leybaert, Z. Molnár, M. E. O'Donnell, J. T. Povlishock, N. R. Saunders, F. Sharp, D. Stanimirovic, R. J. Watts, L. R. Drewes, *Nat. Rev. Neurosci.* **2011**, *12*, 169.
- [28] W. A. Banks, *Nat. Rev. Drug Discovery* **2016**, *15*, 275.
- [29] C. Iadecola, *Nat. Rev. Neurosci.* **2004**, *5*, 347.
- [30] R. M. Ransohoff, P. Kivisakk, G. Kidd, *Nat. Rev. Immunol.* **2003**, *3*, 569.
- [31] M. A. Deli, L. Descamps, M. P. Dehouck, R. Cecchelli, F. Joo, C. S. Abraham, G. Torpier, *J. Neurosci. Res.* **1995**, *41*, 717.
- [32] N. Didier, I. A. Romero, C. Creminon, A. Wijkhuisen, J. Grassi, A. Mabondzo, *J. Neurochem.* **2003**, *86*, 246.
- [33] K. Williams, X. Alvarez, A. A. Lackner, *Glia* **2001**, *36*, 156.
- [34] K. Vass, W. F. Hickey, R. E. Schmidt, H. Lassmann, *Lab. Invest.* **1993**, *69*, 275.

- [35] A. Wong, M. Ye, A. Levy, J. Rothstein, D. Bergles, P. Searson, *Front. Neuroeng.* **2013**, 6, 7.
- [36] A. G. Hudetz, *Microcirculation* **1997**, 4, 233.
- [37] H. Sarin, *J. Angiog. Res.* **2010**, 2, 14.
- [38] S. G. Hormuzdi, M. A. Filippov, G. Mitropoulou, H. Monyer, R. Bruzzone, *Biochim. Biophys. Acta Biomembr.* **2004**, 1662, 113.
- [39] M. W. Brightman, *J. Neurocytol.* **2002**, 31, 595.
- [40] K. K. Jain, *Nanomedicine* **2012**, 7, 1225.
- [41] M. A. Deli, *Biochim. Biophys. Acta Biomembr.* **2009**, 1788, 892.
- [42] J. Bernacki, A. Dobrowolska, K. Nierwińska, A. Malecki, *Pharmacol. Rep.* **2008**, 60, 600.
- [43] H. Wolburg, A. Lippoldt, *Vasc. Pharmacol.* **2002**, 38, 323.
- [44] A. G. Koutsiaris, S. V. Tachmitzi, N. Batis, M. G. Kotoula, C. H. Karabatsas, E. Tsironi, D. Z. Chatzoulis, *Biorheology* **2007**, 44, 375.
- [45] T. Worzfeld, M. Schwaninger, *J. Cereb. Blood Flow Metab.* **2016**, 36, 340.
- [46] S. M. Stamatovic, R. F. Keep, A. V. Andjelkovic, *Curr. Neuropharmacol.* **2008**, 6, 179.
- [47] S. Krol, *J. Controlled Release* **2012**, 164, 145.
- [48] R. A. Yokel, *Nanomed. Nanotechnol. Biol. Med.* **2016**, 12, 2081.
- [49] W. M. Pardridge, *Mol. Biotechnol.* **2005**, 30, 57.
- [50] W. M. Pardridge, *NeuroRx* **2005**, 2, 3.
- [51] E. E. Benarroch, *Neurology* **2012**, 78, 1268.
- [52] Y. Uchida, S. Ohtsuki, Y. Katsukura, C. Ikeda, T. Suzuki, J. Kamiie, T. Terasaki, *J. Neurochem.* **2011**, 117, 333.
- [53] J. Enokizono, H. Kusuhara, A. Ose, A. H. Schinkel, Y. Sugiyama, *Drug Metab. Dispos.* **2008**, 36, 995.
- [54] W. M. Pardridge, *Expert Opin. Biol. Ther.* **2016**, 16, 1455.
- [55] P. J. Gaillard, A. Brink, A. G. de Boer, *Int. Congr. Ser.* **2005**, 1277, 185.
- [56] J. Rip, G. J. Schenk, A. G. De Boer, *Expert Opin. Drug Delivery* **2009**, 6, 227.
- [57] L. A. Opanashuk, R. J. Mark, J. Porter, D. Damm, M. P. Mattson, K. B. Seroogy, *J. Neurosci.* **1999**, 19, 133.
- [58] G. Bu, E. A. Maksymovitch, J. M. Nerbonne, A. L. Schwartz, *J. Biol. Chem.* **1994**, 269, 18521.
- [59] H. Wekerle, M. Schwab, C. Linington, R. Meyermann, *Eur. J. Immunol.* **1986**, 16, 1551.
- [60] H. Wekerle, *J. Infect. Dis.* **2002**, 186, S140.
- [61] G. K. Matsushima, M. Taniike, L. H. Glimcher, M. J. Grusby, J. A. Frelinger, K. Suzuki, J. P. Y. Ting, *Cell* **1994**, 78, 645.
- [62] A. Svenningsson, O. Andersen, M. Edsbacke, S. Stemme, *J. Neuroimmunol.* **1995**, 63, 39.
- [63] M. Schwartz, A. Deczkowska, *Trends Immunol.* **2016**, 37, 668.
- [64] V. H. Perry, P. B. Andersson, *Neuropathol. Appl. Neurobiol.* **1992**, 18, 454.
- [65] A. F. de Vos, M. van Meurs, H. P. Brok, L. A. Boven, R. Q. Hintzen, P. van der Valk, R. Ravid, S. Rensing, L. Boon, A. Bert, *J. Immunol.* **2002**, 169, 5415.
- [66] A. Warth, S. Kröger, H. Wolburg, *Acta Neuropathol.* **2004**, 107, 311.
- [67] R. D. Bell, E. A. Winkler, A. P. Sagare, I. Singh, B. LaRue, R. Deane, B. V. Zlokovic, *Neuron* **2010**, 68, 409.
- [68] R. J. Boado, W. M. Pardridge, *J. Neurochem.* **2002**, 80, 552.

- [69] M. Marroni, N. Marchi, L. Cucullo, N. Abbott, K. Signorelli, D. Janigro, *Curr. Drug Targets* **2003**, *4*, 297.
- [70] R. Kortekaas, K. L. Leenders, J. C. H. van Oostrom, W. Vaalburg, J. Bart, A. Willemsen, N. H. Hendrikse, *Ann. Neurol.* **2005**, *57*, 176.
- [71] O. M. Nwaozuzu, L. A. Sellers, M. A. Barrand, *J. Neurochem.* **2003**, *87*, 1043.
- [72] J. R. Cirrito, R. Deane, A. M. Fagan, M. L. Spinner, M. Parsadanian, M. B. Finn, H. Jiang, J. L. Prior, A. Sagare, K. R. Bales, *J. Clin. Invest.* **2005**, *115*, 3285.
- [73] E. Haroon, C. L. Raison, A. H. Miller, *Neuropsychopharmacology* **2012**, *37*, 137.
- [74] S. Floris, S. R. Ruuls, A. Wierinckx, S. M. A. van der Pol, E. Döpp, P. H. van der Meide, C. D. Dijkstra, H. E. De Vries, *J. Neuroimmunol.* **2002**, *127*, 69.
- [75] V. Moliné-Velázquez, H. Cuervo, V. Vila-Del Sol, M. C. Ortega, D. Clemente, F. de Castro, *Brain Pathol.* **2011**, *21*, 678.
- [76] R. A. Morantz, G. W. Wood, M. Foster, M. Clark, K. Gollahon, *J. Neurosurg.* **1979**, *50*, 305.
- [77] C. Laroche, J. I. Alvarez, A. Prat, *FEBS Lett.* **2011**, *585*, 3770.
- [78] B. V. Zlokovic, J. H. Griffin, *Trends Neurosci.* **2011**, *34*, 198.
- [79] R. Brookmeyer, E. Johnson, K. Ziegler-Graham, H. M. Arrighi, *Alzheimer's Dementia* **2007**, *3*, 186.
- [80] A. Ott, M. M. B. Breteler, F. van Harskamp, J. J. Claus, T. J. M. van der Cammen, D. E. Grobbee, A. Hofman, *BMJ* **1995**, *310*, 970.
- [81] G. C. Román, T. K. Tatemichi, T. Erkinjuntti, J. L. Cummings, J. C. Masdeu, J. H. Garcia, L. Amaducci, J.-M. Orgogozo, A. Brun, A. Hofman, D. M. Moody, M. D. O'Brien, T. Yamaguchi, J. Grafman, B. P. Drayer, D. A. Bennett, M. Fisher, J. Ogata, E. Kokmen, F. Bermejo, P. A. Wolf, P. B. Gorelick, K. L. Bick, A. K. Pajean, M. A. Bell, C. DeCarli, A. Culebras, A. D. Korczyn, J. Bogousslavsky, A. Hartmann, P. Scheinberg, *Neurology* **1993**, *43*, 250.
- [82] A. D. Korczyn, *J. Neurol. Sci.* **2005**, *229*, 3.
- [83] J. Hardy, D. J. Selkoe, *Science* **2002**, *297*, 353.
- [84] G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E. M. Stadlan, *Neurology* **1984**, *34*, 939.
- [85] D. S. Eisenberg, M. R. Sawaya, *Nature* **2017**, *547*, 170.
- [86] D. M. Holtzman, J. C. Morris, A. M. Goate, *Sci. Transl. Med.* **2011**, *3*, 77sr1.
- [87] F. Song, A. Poljak, M. Valenzuela, R. Mayeux, G. A. Smythe, P. S. Sachdev, *J. Alzheimer's Dis.* **2011**, *26*, 365.
- [88] R. Agarwal, C. B. Tripathi, *Int. J. Alzheimer's Dis.* **2011**, *2011*, 503293.
- [89] R. Deane, S. Du Yan, R. K. Subramanian, B. LaRue, S. Jovanovic, E. Hogg, D. Welch, L. Manness, C. Lin, J. Yu, *Nat. Med.* **2003**, *9*, 907.
- [90] S. Du Yan, X. Chen, J. Fu, M. Chen, *Nature* **1996**, *382*, 685.
- [91] K. G. Mawuenyega, W. Sigurdson, V. Ovod, L. Munsell, T. Kasten, J. C. Morris, K. E. Yarasheski, R. J. Bateman, *Science* **2010**, *330*, 1774.
- [92] R. A. Marr, D. M. Hafez, *Front. Aging Neurosci.* **2014**, *6*, 187.
- [93] J. S. Miners, S. Baig, J. Palmer, L. E. Palmer, P. G. Kehoe, S. Love, *Brain Pathol.* **2008**, *18*, 240.
- [94] R. A. Marr, E. Rockenstein, A. Mukherjee, M. S. Kindy, L. B. Hersh, F. H. Gage, I. M. Verma, E. Masliah, *J. Neurosci.* **2003**, *23*, 1992.
- [95] N. Iwata, S. Tsubuki, Y. Takaki, K. Watanabe, M. Sekiguchi, E. Hosoki, M. Kawashima-Morishima, H.-J. Lee, E. Hama, Y. Sekine-Aizawa, *Nat. Med.* **2000**, *6*, 143.

- [96] I. Singh, A. P. Sagare, M. Coma, D. Perlmutter, R. Gelein, R. D. Bell, R. J. Deane, E. Zhong, M. Parisi, J. Ciszewski, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 14771.
- [97] R. J. Bateman, L. Y. Munsell, J. C. Morris, R. Swarm, K. E. Yarasheski, D. M. Holtzman, *Nat. Med.* **2006**, *12*, 856.
- [98] K. J. Barnham, C. L. Masters, A. I. Bush, *Nat. Rev. Drug Discovery* **2004**, *3*, 205.
- [99] A. I. Bush, *Trends Neurosci.* **2003**, *26*, 207.
- [100] A. I. Bush, W. H. Pettingell, G. Multhaup, M. Paradis, J.-P. Vonsattel, J. F. Gusella, K. Beyreuther, C. L. Masters, R. E. Tanzi, *Science* **1994**, *265*, 1464.
- [101] X. Huang, C. S. Atwood, M. A. Hartshorn, G. Multhaup, L. E. Goldstein, R. C. Scarpa, M. P. Cuajungco, D. N. Gray, J. Lim, R. D. Moir, R. E. Tanzi, A. I. Bush, *Biochemistry* **1999**, *38*, 7609.
- [102] R. A. Cherny, C. S. Atwood, M. E. Xilinas, D. N. Gray, W. D. Jones, C. A. McLean, K. J. Barnham, I. Volitakis, F. W. Fraser, Y.-S. Kim, *Neuron* **2001**, *30*, 665.
- [103] D. J. Selkoe, J. Hardy, *EMBO Mol. Med.* **2016**, *8*, 595.
- [104] R. E. Tanzi, L. Bertram, *Cell* **2005**, *120*, 545.
- [105] L. Buée, P. R. Hof, C. Bouras, A. Delacourte, D. P. Perl, J. H. Morrison, H. M. Fillit, *Acta Neuropathol.* **1994**, *87*, 469.
- [106] M. A. Erickson, W. A. Banks, *J. Cereb. Blood Flow Metab.* **2013**, *33*, 1500.
- [107] Y. Deschaintre, F. Richard, D. Leys, F. Pasquier, *Neurology* **2009**, *73*, 674.
- [108] S. A. Farr, M. A. Erickson, M. L. Niehoff, W. A. Banks, J. E. Morley, *J. Alzheimer's Dis.* **2014**, *40*, 1005.
- [109] H. Keren-Shaul, A. Spinrad, A. Weiner, O. Matcovitch-Natan, R. Dvir-Szternfeld, T. K. Ulland, E. David, K. Baruch, D. Lara-Astaiso, B. Toth, S. Itzkovitz, M. Colonna, M. Schwartz, I. Amit, *Cell* **2017**, *169*, 1276.
- [110] G. Leinenga, J. Götz, *Sci. Transl. Med.* **2015**, *7*, 278ra33.
- [111] B. D. James, D. A. Bennett, P. A. Boyle, S. Leurgans, J. A. Schneider, *JAMA* **2012**, *307*, 1798.
- [112] C. Brown, *Nature* **2016**, *540*, S4.
- [113] C. Schmidt, *Nature* **2016**, *540*, S2.
- [114] L. Steinman, *Neuron* **1999**, *24*, 511.
- [115] C. Du, X. Xie, *Cell Res.* **2012**, *22*, 1108.
- [116] M. Mahmoudi, M. A. Sahraian, M. A. Shokrgozar, S. Laurent, *ACS Chem. Neurosci.* **2011**, *2*, 118.
- [117] L. C. Hudson, D. C. Bragg, M. B. Tompkins, R. B. Meeker, *Brain Res.* **2005**, *1058*, 148.
- [118] C. Y. Wu, H. L. Hsieh, C. C. Sun, C. M. Yang, *Glia* **2009**, *57*, 1775.
- [119] D. Leppert, J. Ford, G. Stabler, C. Grygar, C. Lienert, S. Huber, K. M. Miller, S. L. Hauser, L. Kappos, *Brain* **1998**, *121*, 2327.
- [120] S. Hofmann, H. Grasberger, P. Jung, M. Bidlingmaier, J. Vlotides, O. E. Janssen, R. Landgraf, *Eur. J. Med. Res.* **2002**, *7*, 171.
- [121] S. Agrawal, P. Anderson, M. Durbeej, N. van Rooijen, F. Ivars, G. Opdenakker, L. M. Sorokin, *J. Exp. Med.* **2006**, *203*, 1007.
- [122] M. Sixt, B. Engelhardt, F. Pausch, R. Hallmann, O. Wendler, L. M. Sorokin, *J. Cell Biol.* **2001**, *153*, 933.
- [123] B. Cannella, C. S. Raine, *Ann. Neurol.* **1995**, *37*, 424.
- [124] U. Traugott, E. L. Reinherz, C. S. Raine, *Science* **1983**, *219*, 308.
- [125] R. Cabezas, M. Ávila, J. Gonzalez, R. S. El-Bachá, E. Báez, L. M. García-Segura, J. C. Jurado Coronel, F. Capani, G. P. Cardona-Gomez, G. E. Barreto, *Front. Cell. Neurosci.* **2014**, *8*, 211.

- [126] B. S. Desai, A. J. Monahan, P. M. Carvey, B. Hendey, *Cell Transplant.* **2007**, *16*, 285.
- [127] H. Braak, K. Del Tredici, U. Rüb, R. A. I. De Vos, E. N. H. J. Steur, E. Braak, *Neurobiol. Aging* **2003**, *24*, 197.
- [128] C. Zarow, S. A. Lyness, J. A. Mortimer, H. C. Chui, *Arch. Neurol.* **2003**, *60*, 337.
- [129] M. B. H. Youdim, Y. S. Bakhle, *Br. J. Pharmacol.* **2006**, *147*, S287.
- [130] GBD 2015 Mortality and Causes of Death Collaborators, *Lancet* **2016**, 388, 1459.
- [131] Z. Corbyn, *Nature* **2014**, *510*, S2.
- [132] R. Jin, G. Yang, G. Li, *Neurobiol. Dis.* **2010**, *38*, 376.
- [133] D. W. Choi, S. M. Rothman, *Annu. Rev. Neurosci.* **1990**, *13*, 171.
- [134] U. Dirnagl, in *Neuroinflammation in Stroke*, (Eds: U. Dirnagl, B. Elger), Springer-Verlag Berlin Heidelberg, Berlin, Heidelberg, Germany **2004**, Ch. 5.
- [135] L. Belayev, R. Busto, W. Zhao, M. D. Ginsberg, *Brain Res.* **1996**, *739*, 88.
- [136] Y. I. Yang, E. Y. Estrada, J. F. Thompson, W. Liu, G. A. Rosenberg, *J. Cereb. Blood Flow Metab.* **2007**, *27*, 697.
- [137] N. Schmid-Brunclik, C. Bürgi-Taboada, X. Antoniou, M. Gassmann, O. O. Ogunshola, *Am. J. Physiol.: Regul. Integr. Comp. Physiol.* **2008**, *295*, R864.
- [138] J. D. Huber, R. D. Egleton, T. P. Davis, *Trends Neurosci.* **2001**, *24*, 719.
- [139] A. L. Betz, R. F. Keep, M. E. Beer, X.-D. Ren, *J. Cereb. Blood Flow Metab.* **1994**, *14*, 29.
- [140] A. Spudich, E. Kilic, H. Xing, Ü. Kilic, K. M. Rentsch, H. Wunderli-Allenspach, C. L. Bassetti, D. M. Hermann, *Nat. Neurosci.* **2006**, *9*, 487.
- [141] N. D. Tran, J. Correale, S. S. Schreiber, M. Fisher, *Stroke* **1999**, *30*, 1671.
- [142] E. H. Lo, T. Dalkara, M. A. Moskowitz, *Nat. Rev. Neurosci.* **2003**, *4*, 399.
- [143] A. R. Young, C. Ali, A. Duretête, D. Vivien, *J. Neurochem.* **2007**, *103*, 1302.
- [144] W. Hacke, M. Kaste, E. Bluhmki, M. Brozman, A. Dávalos, D. Guidetti, V. Larrue, K. R. Lees, Z. Medeghri, T. Machnig, *N. Engl. J. Med.* **2008**, *359*, 1317.
- [145] O. Nicole, F. Docagne, C. Ali, I. Margail, P. Carmeliet, E. T. MacKenzie, D. Vivien, A. Buisson, *Nat. Med.* **2001**, *7*, 59.
- [146] W. M. Pardridge, *Pharm. Res.* **2007**, *24*, 1733.
- [147] A. Tao, L. Tao, C. Nicholson, *J. Theor. Biol.* **2005**, *234*, 525.
- [148] R. G. Blasberg, C. Patlak, J. D. Fenstermacher, *J. Pharmacol. Exp. Ther.* **1975**, *195*, 73.
- [149] R. K. Jain, *J. Natl. Cancer Inst.* **1989**, *81*, 570.
- [150] E. J. Mufson, J. S. Kroin, Y. T. Liu, T. Sobriela, R. D. Penn, J. A. Miller, J. H. Kordower, *Neuroscience* **1996**, *71*, 179.
- [151] L. K. Fung, M. Shin, B. Tyler, H. Brem, W. M. Saltzman, *Pharm. Res.* **1996**, *13*, 671.
- [152] M. Y. Chen, A. Hoffer, P. F. Morrison, J. F. Hamilton, J. Hughes, K. S. Schlageter, J. Lee, B. R. Kelly, E. H. Oldfield, *J. Neurosurg.* **2005**, *103*, 311.
- [153] S. J. Allen, J. J. Watson, D. K. Shoemark, N. U. Barua, N. K. Patel, *Pharmacol. Ther.* **2013**, *138*, 155.
- [154] R. Stupp, W. P. Mason, M. J. Van Den Bent, M. Weller, B. Fisher, M. J. B. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, *N. Engl. J. Med.* **2005**, *352*, 987.
- [155] J. Zhou, T. R. Patel, R. W. Sirianni, G. Strohhahn, M.-Q. Zheng, N. Duong, T. Schafbauer, A. J. Huttner, Y. Huang, R. E. Carson, Y. Zhang, D. J. Sullivan, J. M. Piepmeyer, W. M. Saltzman, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 11751.
- [156] C. L. Nilsson, K. A. Cunningham, *ACS Chem. Neurosci.* **2018**, *9*, 5.
- [157] R. Raghavan, M. L. Brady, M. I. Rodríguez-Ponce, A. Hartlep, C. Pedain, J. H. Sampson, *Neurosurg. Focus* **2006**, *20*, E12.
- [158] M. I. Papisov, V. V. Belov, K. S. Gannon, *Mol. Pharm.* **2013**, *10*, 1522.

- [159] Y. Okuda, S. Sakoda, H. Fujimura, S. Nagata, T. Yanagihara, C. C. A. Bernard, *Biochem. Biophys. Res. Commun.* **2000**, 275, 164.
- [160] J. W. Maina, J. Cui, M. Björnalm, A. K. Wise, R. K. Shepherd, F. Caruso, *Biomacromolecules* **2014**, 15, 4146.
- [161] Y. Wang, A. K. Wise, J. Tan, J. W. Maina, R. K. Shepherd, F. Caruso, *Small* **2014**, 10, 4244.
- [162] A. K. Wise, L. N. Gillespie, *J. Neural Eng.* **2012**, 9, 065002.
- [163] S. Boshier, R. Warren, *Proc. R. Soc. London Ser. B* **1968**, 171, 227.
- [164] O. Sterkers, E. Ferrary, C. Amiel, *Physiol. Rev.* **1988**, 68, 1083.
- [165] X. Zhang, G. Chen, L. Wen, F. Yang, A. I. Shao, X. Li, W. Long, L. Mu, *Eur. J. Pharm. Sci.* **2013**, 48, 595.
- [166] G. Chen, X. Zhang, F. Yang, L. Mu, *Curr. Drug Metab.* **2010**, 11, 886.
- [167] G. Chen, S. X. Hou, P. Hu, M. Z. Jin, J. Liu, *Acta Pharm. Sin. B* **2007**, 42, 1102.
- [168] S. V. Dhuria, L. R. Hanson, W. H. Frey, *J. Pharm. Sci.* **2010**, 99, 1654.
- [169] J. J. Lochhead, R. G. Thorne, *Adv. Drug Delivery Rev.* **2012**, 64, 614.
- [170] H. Xia, X. Gao, G. Gu, Z. Liu, N. Zeng, Q. Hu, Q. Song, L. Yao, Z. Pang, X. Jiang, J. Chen, H. Chen, *Biomaterials* **2011**, 32, 9888.
- [171] L. Biddlestone-Thorpe, N. Marchi, K. Guo, C. Ghosh, D. Janigro, K. Valerie, H. Yang, *Adv. Drug Delivery Rev.* **2012**, 64, 605.
- [172] T. Kanazawa, H. Taki, K. Tanaka, Y. Takashima, H. Okada, *Pharm. Res.* **2011**, 28, 2130.
- [173] L. Kozlovskaya, M. Abou-Kaoud, D. Stepensky, *J. Controlled Release* **2014**, 189, 133.
- [174] N. M. Smith, I. Gachulinova, D. Ho, C. Bailey, C. A. Bartlett, M. Norret, J. Murphy, A. Buckley, P. J. Rigby, M. J. House, T. St. Pierre, M. Fitzgerald, K. S. Iyer, S. A. Dunlop, *Sci. Rep.* **2016**, 6, 22595.
- [175] H. Pajouhesh, G. R. Lenz, *NeuroRx* **2005**, 2, 541.
- [176] C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv. Drug Delivery Rev.* **2012**, 64, 4.
- [177] K. A. Witt, T. J. Gillespie, J. D. Huber, R. D. Egleton, T. P. Davis, *Peptides* **2001**, 22, 2329.
- [178] L. Juillerat-Jeanneret, *Drug Discovery Today* **2008**, 13, 1099.
- [179] E. M. Cornford, D. Young, J. W. Paxton, G. J. Finlay, W. R. Wilson, W. M. Pardridge, *Cancer Res.* **1992**, 52, 138.
- [180] W. M. Pardridge, *Adv. Drug Delivery Rev.* **1999**, 36, 299.
- [181] J. Kreuter, *Adv. Drug Delivery Rev.* **2001**, 47, 65.
- [182] S. Muro, M. Koval, V. Muzykantov, *Curr. Vasc. Pharmacol.* **2004**, 2, 281.
- [183] G. Sahay, D. Y. Alakhova, A. V. Kabanov, *J. Controlled Release* **2010**, 145, 182.
- [184] L. M. Bareford, P. W. Swaan, *Adv. Drug Delivery Rev.* **2007**, 59, 748.
- [185] R. Villaseñor, M. Schilling, J. Sundaresan, Y. Lutz, L. Collin, *Cell Rep.* **2017**, 21, 3256.
- [186] A. S. Haqqani, C. E. Delaney, E. Brunette, E. Baumann, G. K. Farrington, W. Sisk, J. Eldredge, W. Ding, T.-L. Tremblay, D. B. Stanimirovic, *J. Cereb. Blood Flow Metab.* **2017**, 37, 1.
- [187] M. Bartsch, A. H. Weeke-Klimp, D. K. F. Meijer, G. L. Scherphof, J. A. A. M. Kamps, *J. Liposome Res.* **2005**, 15, 59.
- [188] A. Verma, F. Stellacci, *Small* **2010**, 6, 12.
- [189] R. G. Parton, M. T. Howes, *J. Cell Biol.* **2010**, 191, 439.

- [190] L. I. Selby, C. M. Cortez-Jugo, G. K. Such, A. P. R. Johnston, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2017**, *9*, e1452.
- [191] A. Hayer, M. Stoeber, D. Ritz, S. Engel, H. H. Meyer, A. Helenius, *J. Cell Biol.* **2010**, *191*, 615.
- [192] S. Wohlfart, S. Gelperina, J. Kreuter, *J. Controlled Release* **2012**, *161*, 264.
- [193] A. R. Jones, E. V. Shusta, *Pharm. Res.* **2007**, *24*, 1759.
- [194] A. G. De Boer, P. J. Gaillard, *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 323.
- [195] Z. H. Wang, Z. Y. Wang, C. S. Sun, C. Y. Wang, T. Y. Jiang, S. L. Wang, *Biomaterials* **2010**, *31*, 908.
- [196] F. Hervé, N. Ghinea, J.-M. Scherrmann, *AAPS J.* **2008**, *10*, 455.
- [197] M. Zorko, Ü. Langel, *Adv. Drug Delivery Rev.* **2005**, *57*, 529.
- [198] U. Bickel, T. Yoshikawa, W. M. Pardridge, *Adv. Drug Delivery Rev.* **2001**, *46*, 247.
- [199] E. V. Batrakova, H. E. Gendelman, A. V. Kabanov, *Expert Opin. Drug Delivery* **2011**, *8*, 415.
- [200] S. Jain, V. Mishra, P. Singh, P. K. Dubey, D. K. Saraf, S. P. Vyas, *Int. J. Pharm.* **2003**, *261*, 43.
- [201] J. Qin, D. Chen, H. Hu, Q. Cui, M. Qiao, B. Chen, *Chem. Pharm. Bull.* **2007**, *55*, 1192.
- [202] W. V. Rodriguez, P. H. Pritchard, M. J. Hope, *Biochim. Biophys. Acta Biomembr.* **1993**, *1153*, 9.
- [203] R. H. Fang, Y. Jiang, J. C. Fang, L. Zhang, *Biomaterials* **2017**, *128*, 69.
- [204] A. Parodi, N. Quattrocchi, A. L. Van De Ven, C. Chiappini, M. Evangelopoulos, J. O. Martinez, B. S. Brown, S. Z. Khaled, I. K. Yazdi, M. V. Enzo, *Nat. Nano.* **2013**, *8*, 61.
- [205] C.-M. J. Hu, L. Zhang, S. Aryal, C. Cheung, R. H. Fang, L. Zhang, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 10980.
- [206] R. H. Adamson, J. F. Lenz, X. Zhang, G. N. Adamson, S. Weinbaum, F. E. Curry, *J. Physiol.* **2004**, *557*, 889.
- [207] S. I. Rapoport, K. Ohno, W. R. Fredericks, K. D. Pettigrew, *Brain Res.* **1978**, *150*, 653.
- [208] T. Siegal, R. Rubinstein, F. Bokstein, A. Schwartz, A. Lossos, E. Shalom, R. Chisin, J. M. Gomori, *J. Neurosurg.* **2000**, *92*, 599.
- [209] R. F. Barth, W. Yang, R. T. Bartus, M. L. Moeschberger, J. H. Goodman, *Neurosurgery* **1999**, *44*, 351.
- [210] D. F. Emerich, P. Snodgrass, R. Dean, M. Agostino, B. Hasler, M. Pink, H. Xiong, B. S. Kim, R. T. Bartus, *Br. J. Cancer* **1999**, *80*, 964.
- [211] N. J. Abbott, *Cell. Mol. Neurobiol.* **2000**, *20*, 131.
- [212] K. Hynynen, N. McDannold, N. A. Sheikov, F. A. Jolesz, N. Vykhodtseva, *NeuroImage* **2005**, *24*, 12.
- [213] K. Hynynen, N. McDannold, N. Vykhodtseva, F. A. Jolesz, *Radiology* **2001**, *220*, 640.
- [214] M. Kinoshita, N. McDannold, F. A. Jolesz, K. Hynynen, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 11719.
- [215] K. Hynynen, N. McDannold, N. Vykhodtseva, S. Raymond, R. Weissleder, F. A. Jolesz, N. Sheikov, *J. Neurosurg.* **2006**, *105*, 445.
- [216] M. Aryal, C. D. Arvanitis, P. M. Alexander, N. McDannold, *Adv. Drug Delivery Rev.* **2014**, *72*, 94.
- [217] H.-L. Liu, C.-H. Fan, C.-Y. Ting, C.-K. Yeh, *Theranostics* **2014**, *4*, 432.
- [218] K. Hynynen, F. A. Jolesz, *Ultrasound Med. Biol.* **1998**, *24*, 275.
- [219] C. Sierra, C. Acosta, C. Chen, S.-Y. Wu, M. E. Karakatsani, M. Bernal, E. E. Konofagou, *J. Cereb. Blood Flow Metab.* **2017**, *37*, 1236.

- [220] E. E Konofagou, Y.-S. Tunga, J. Choia, T. Deffieuxa, B. Baseria, F. Vlachosa, *Curr. Pharm. Biotechnol.* **2012**, *13*, 1332.
- [221] G. Samiotaki, F. Vlachos, Y. S. Tung, E. E. Konofagou, *Magn. Reson. Med.* **2012**, *67*, 769.
- [222] T. Lammers, P. Koczera, S. Fokong, F. Gremse, J. Ehling, M. Vogt, A. Pich, G. Storm, M. Van Zandvoort, F. Kiessling, *Adv. Funct. Mater.* **2015**, *25*, 36.
- [223] E. Moriyama, M. Salcman, R. D. Broadwell, *Surg. Neurol.* **1991**, *35*, 177.
- [224] J. H. Merritt, A. F. Chamness, S. J. Allen, *Radiat. Environ. Biophys.* **1978**, *15*, 367.
- [225] L. G. Salford, A. Brun, K. Stureson, J. L. Eberhardt, B. R. R. Persson, *Microsc. Res. Tech.* **1994**, *27*, 535.
- [226] Y.-C. Kuo, C.-Y. Kuo, *Int. J. Pharm.* **2008**, *351*, 271.
- [227] E. M. Kemper, W. Boogerd, I. Thuis, J. H. Beijnen, O. van Tellingen, *Cancer Treat. Rev.* **2004**, *30*, 415.
- [228] M. Haluska, M. L. Anthony, *Clin. J. Oncol. Nurs.* **2004**, *8*, 263.
- [229] W. A. Banks, *Adv. Drug Delivery Rev.* **2012**, *64*, 629.
- [230] W. Löscher, H. Potschka, *Nat. Rev. Neurosci.* **2005**, *6*, 591.
- [231] C. Funke, M. Farr, B. Werner, S. Dittmann, K. Überla, C. Piper, K. Niehaus, D. Horstkotte, *J. Gen. Virol.* **2010**, *91*, 1959.
- [232] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, *Cancer Res.* **1981**, *41*, 1967.
- [233] W. T. Beck, M. C. Cirtain, C. J. Glover, R. L. Felsted, A. R. Safa, *Biochem. Biophys. Res. Commun.* **1988**, *153*, 959.
- [234] J. Van Asperen, O. Van Tellingen, A. Sparreboom, A. H. Schinkel, P. Borst, W. J. Nooijen, J. H. Beijnen, *Br. J. Cancer* **1997**, *76*, 1181.
- [235] H. A. Bardelmeijer, J. H. Beijnen, K. R. Brouwer, H. Rosing, W. J. Nooijen, J. H. M. Schellens, O. van Tellingen, *Clin. Cancer. Res.* **2000**, *6*, 4416.
- [236] E. Fox, S. E. Bates, *Expert Rev. Anticancer Ther.* **2007**, *7*, 447.
- [237] M. Werle, *Pharm. Res.* **2008**, *25*, 500.
- [238] J. C. Kalvass, J. W. Polli, D. L. Bourdet, B. Feng, S. M. Huang, X. Liu, Q. R. Smith, L. K. Zhang, M. J. Zamek-Gliszczynski, C. International Transporter, *Clin. Pharmacol. Ther.* **2013**, *94*, 80.
- [239] Y. Yan, M. Björnmalm, F. Caruso, *ACS Nano* **2013**, *7*, 9512.
- [240] G. Dumortier, J. L. Grossiord, F. Agnely, J. C. Chaumeil, *Pharm. Res.* **2006**, *23*, 2709.
- [241] P. Alexandridis, T. A. Hatton, *Colloids Surf. A* **1995**, *96*, 1.
- [242] E. V. Batrakova, A. V. Kabanov, *J. Controlled Release* **2008**, *130*, 98.
- [243] E. V. Batrakova, S. Li, Y. Li, V. Y. Alakhov, A. V. Kabanov, *Pharm. Res.* **2004**, *21*, 2226.
- [244] E. V. Batrakova, S. Li, D. W. Miller, A. V. Kabanov, *Pharm. Res.* **1999**, *16*, 1366.
- [245] E. V. Batrakova, S. Li, S. V. Vinogradov, V. Y. Alakhov, D. W. Miller, A. V. Kabanov, *J. Pharmacol. Exp. Ther.* **2001**, *299*, 483.
- [246] E. V. Batrakova, D. W. Miller, S. Li, V. Y. Alakhov, A. V. Kabanov, W. F. Elmquist, *J. Pharmacol. Exp. Ther.* **2001**, *296*, 551.
- [247] A. V. Kabanov, E. V. Batrakova, D. W. Miller, *Adv. Drug Delivery Rev.* **2003**, *55*, 151.
- [248] E. V. Batrakova, S. Li, V. Y. Alakhov, D. W. Miller, A. V. Kabanov, *J. Pharmacol. Exp. Ther.* **2003**, *304*, 845.
- [249] S. Danson, D. Ferry, V. Alakhov, J. Margison, D. Kerr, D. Jowle, M. Brampton, G. Halbert, M. Ranson, *Br. J. Cancer* **2004**, *90*, 2085.
- [250] J. W. Valle, A. Armstrong, C. Newman, V. Alakhov, G. Pietrzynski, J. Brewer, S. Campbell, P. Corrie, E. K. Rowinsky, M. Ranson, *Invest. New Drugs* **2011**, *29*, 1029.

- [251] D. J. Begley, *Curr. Pharm. Des.* **2004**, *10*, 1295.
- [252] C. Hansch, J. P. Björkroth, A. Leo, *J. Pharm. Sci.* **1987**, *76*, 663.
- [253] P. D. Leeson, A. M. Davis, *J. Med. Chem.* **2004**, *47*, 6338.
- [254] H. van de Waterbeemd, G. Camenisch, G. Folkers, J. R. Chretien, O. A. Raevsky, *J. Drug Targeting* **1998**, *6*, 151.
- [255] J. Kelder, P. D. J. Grootenhuis, D. M. Bayada, L. P. C. Delbressine, J.-P. Ploemen, *Pharm. Res.* **1999**, *16*, 1514.
- [256] D. Pan, M. Iyer, J. Liu, Y. Li, A. J. Hopfinger, *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 2083.
- [257] D. E. Clark, *Drug Discovery Today* **2003**, *8*, 927.
- [258] J. H. Lin, A. D. Rodrigues, in *Pharmacokinetic Optimization in Drug Research: Biological, Physicochemical and Computational Strategies*, (Eds: B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy), Wiley-VCH, Weinheim, Germany **2001**, Ch. 3.
- [259] T. J. Raub, B. S. Lutzke, P. K. Andrus, G. A. Sawada, B. A. Staton, in *Optimizing the "Drug-Like" Properties of Leads in Drug Discovery*, (Eds: R. T. Borchardt, E. H. Kerns, M. J. Hageman, D. R. Thakker, J. L. Stevens), Springer, New York, NY, USA **2006**, Ch. 16.
- [260] W. S. Redfern, L. Carlsson, A. S. Davis, W. G. Lynch, I. MacKenzie, S. Palethorpe, P. K. S. Siegl, I. Strang, A. T. Sullivan, R. Wallis, *Cardiovasc. Res.* **2003**, *58*, 32.
- [261] B. Wang, W. Wang, H. Zhang, D. Shan, K. Nimkar, O. Gudmundsson, S. Gangwar, T. Siahhaan, R. T. Borchardt, *J. Pept. Res.* **1999**, *53*, 370.
- [262] T. J. Abbruscato, S. A. Williams, A. Misicka, A. W. Lipkowski, V. J. Hruby, T. P. Davis, *J. Pharmacol. Exp. Ther.* **1996**, *276*, 1049.
- [263] S. J. Weber, T. J. Abbruscato, E. A. Brownson, A. W. Lipkowski, R. Polt, A. Misicka, R. C. Haaseth, H. Bartosz, V. J. Hruby, T. P. Davis, *J. Pharmacol. Exp. Ther.* **1993**, *266*, 1649.
- [264] D. Brewster, P. W. Dettmar, G. Metcalf, *Neuropharmacology* **1981**, *20*, 497.
- [265] P. S. Dobbin, R. C. Hider, A. D. Hall, P. D. Taylor, P. Sarpong, J. B. Porter, G. Xiao, D. van der Helm, *J. Med. Chem.* **1993**, *36*, 2448.
- [266] W. A. Banks, A. J. Kastin, D. H. Coy, E. Angulo, *Brain Res. Bull.* **1986**, *17*, 155.
- [267] D. L. Greene, V. S. Hau, T. J. Abbruscato, H. Bartosz, A. Misicka, A. W. Lipkowski, S. Hom, T. J. Gillespie, V. J. Hruby, T. P. Davis, *J. Pharmacol. Exp. Ther.* **1996**, *277*, 1366.
- [268] V. E. Shashoua, J. N. Jacob, R. Ridge, A. Campbell, R. J. Baldessarini, *J. Med. Chem.* **1984**, *27*, 659.
- [269] J. A. Arnott, S. L. Planey, *Expert Opin. Drug Discovery* **2012**, *7*, 863.
- [270] D. A. Price, J. Blagg, L. Jones, N. Greene, T. Wager, *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 921.
- [271] V. J. Stella, *Expert Opin. Ther. Pat.* **2004**, *14*, 277.
- [272] R. Rais, A. Jančařík, L. Tenora, M. Nedelcovych, J. Alt, J. Englert, C. Rojas, A. Le, A. Elgogary, J. Tan, L. Monincová, K. Pate, R. Adams, D. Ferraris, J. Powell, P. Majer, B. S. Slusher, *J. Med. Chem.* **2016**, *59*, 8621.
- [273] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Järvinen, J. Savolainen, *Nat. Rev. Drug Discovery* **2008**, *7*, 255.
- [274] G. B. Magill, W. P. L. Myers, H. C. Reilly, R. C. Putnam, J. W. Magill, M. P. Sykes, G. C. Escher, D. A. Karnofsky, J. H. Burchenal, *Cancer* **1957**, *10*, 1138.
- [275] V. Stella, R. Borchardt, M. Hageman, R. Oliyai, H. Maag, J. Tilley, *Prodrugs: Challenges and Rewards*, Springer Science & Business Media, New York, NJ, USA **2007**.

- [276] S. Ohtsuki, T. Terasaki, *Pharm. Res.* **2007**, *24*, 1745.
- [277] D. Schrama, R. A. Reisfeld, J. C. Becker, *Nat. Rev. Drug Discovery* **2006**, *5*, 147.
- [278] R. Gabathuler, *Neurobiol. Dis.* **2010**, *37*, 48.
- [279] Y. J. Yu, Y. Zhang, M. Kenrick, K. Hoyte, W. Luk, Y. Lu, J. Atwal, J. M. Elliott, S. Prabhu, R. J. Watts, *Sci. Transl. Med.* **2011**, *3*, 84ra44.
- [280] C. Sun, J. S. H. Lee, M. Zhang, *Adv. Drug Delivery Rev.* **2008**, *60*, 1252.
- [281] K. A. Foon, *Cancer Res.* **1989**, *49*, 1621.
- [282] A. L. Nelson, E. Dhimolea, J. M. Reichert, *Nat. Rev. Drug Discovery* **2010**, *9*, 767.
- [283] R. De Pascalis, M. Iwahashi, M. Tamura, E. A. Padlan, N. R. Gonzales, A. D. Santos, M. Giuliano, P. Schuck, J. Schlom, S. V. S. Kashmiri, *J. Immunol.* **2002**, *169*, 3076.
- [284] P. C. Caron, J. G. Jurcic, A. M. Scott, R. D. Finn, C. R. Divgi, M. C. Graham, I. M. Jureidini, G. Sgouros, D. Tyson, L. J. Old, *Blood* **1994**, *83*, 1760.
- [285] M. Harmsen, H. De Haard, *Appl. Microbiol. Biotechnol.* **2007**, *77*, 13.
- [286] A. Ghannam, S. Kumari, S. Muyldermans, A. Q. Abbady, *Plant Mol. Biol.* **2015**, *87*, 355.
- [287] R. L. Stanfield, H. Dooley, M. F. Flajnik, I. A. Wilson, *Science* **2004**, *305*, 1770.
- [288] J. Nilvebrant, M. Åstrand, M. Georgieva-Kotseva, M. Björnmalm, J. Löfblom, S. Hober, *PLoS One* **2014**, *9*, e103094.
- [289] Y. L. Boersma, A. Plückthun, *Curr. Opin. Biotechnol.* **2011**, *22*, 849.
- [290] J. Löfblom, F. Y. Frejld, S. Ståhl, *Curr. Opin. Biotechnol.* **2011**, *22*, 843.
- [291] M. T. Stumpp, P. Amstutz, *Curr. Opin. Drug Discovery Dev.* **2007**, *10*, 153.
- [292] P. Varamini, I. Toth, *Front. Pharmacol.* **2013**, *4*, 1.
- [293] A. L. Becker, A. P. R. Johnston, F. Caruso, *Small* **2010**, *6*, 1836.
- [294] M. Wilchek, E. A. Bayer, *Methods Enzymol.* **1990**, *184*, 5.
- [295] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056; *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 2004
- [296] H. Nandivada, X. Jiang, J. Lahann, *Adv. Mater.* **2007**, *19*, 2197.
- [297] K. Kettler, K. Veltman, D. van de Meent, A. van Wezel, A. J. Hendriks, *Environ. Toxicol. Chem.* **2014**, *33*, 481.
- [298] B. D. Chithrani, A. A. Ghazani, W. C. W. Chan, *Nano Lett.* **2006**, *6*, 662.
- [299] F. Lu, S. H. Wu, Y. Hung, C. Y. Mou, *Small* **2009**, *5*, 1408.
- [300] W. Jiang, B. Y. Kim, J. T. Rutka, W. C. Chan, *Nat. Nano.* **2008**, *3*, 145.
- [301] F. Osaki, T. Kanamori, S. Sando, T. Sera, Y. Aoyama, *J. Am. Chem. Soc.* **2004**, *126*, 6520.
- [302] S.-H. Wang, C.-W. Lee, A. Chiou, P.-K. Wei, *J. Nanobiotechnol.* **2010**, *8*, 33.
- [303] S. Bhaskar, F. Tian, T. Stoeger, W. Kreyling, J. M. de la Fuente, V. Grazú, P. Borm, G. Estrada, V. Ntziachristos, D. Razansky, *Part. Fibre Toxicol.* **2010**, *7*, 3.
- [304] S. Fiering, *Nat. Nano.* **2017**, *12*, 615.
- [305] S. Osuka, E. G. Van Meir, *Nat. Nano.* **2017**, *12*, 616.
- [306] J. J. Richardson, M. Björnmalm, F. Caruso, *Science* **2015**, *348*, aaa2491.
- [307] V. P. Torchilin, *Nat. Rev. Drug Discovery* **2005**, *4*, 145.
- [308] S.-Y. Seong, P. Matzinger, *Nat. Rev. Immunol.* **2004**, *4*, 469.
- [309] D. T. Mlynarczyk, T. Kocki, T. Goslinski, in *Nanostructured Materials – Fabrication to Applications*, (Ed: M. S. Seehra), InTech, Rijeka, Croatia **2017**, Ch. 6.
- [310] G. Suffredini, J. E. East, L. M. Levy, *AJNR, Am. J. Neuroradiol.* **2014**, *35*, 1246.
- [311] M. S. Webb, T. O. Harasym, D. Masin, M. B. Bally, L. D. Mayer, *Br. J. Cancer* **1995**, *72*, 896.
- [312] N. Grimaldi, F. Andrade, N. Segovia, L. Ferrer-Tasies, S. Sala, J. Veciana, N. Ventosa, *Chem. Soc. Rev.* **2016**, *45*, 6520.

- [313] F. Szoka Jr, D. Papahadjopoulos, *Annu. Rev. Biophys. Bioeng.* **1980**, *9*, 467.
- [314] J. Conriot, J. M. Silva, J. G. Fernandes, L. C. Silva, R. Gaspar, S. Brocchini, H. F. Florindo, T. S. Barata, *Front. Chem.* **2014**, *2*, 105.
- [315] L. Ciani, S. Ristori, A. Salvati, L. Calamai, G. Martini, *Biochim. Biophys. Acta Biomembr.* **2004**, *1664*, 70.
- [316] M. C. de Lima, S. Neves, A. Filipe, N. Duzgunes, S. Simoes, *Curr. Med. Chem.* **2003**, *10*, 1221.
- [317] D. A. Hume, *Curr. Opin. Immunol.* **2006**, *18*, 49.
- [318] K. Mehta, G. Lopez-Berestein, E. M. Hersh, R. L. Juliano, *J. Reticuloendothel. Soc.* **1982**, *32*, 155.
- [319] A. Katragadda, R. Bridgman, G. Betageri, *Cell. Mol. Biol. Lett.* **2000**, *5*, 483.
- [320] A. Raz, C. Bucana, W. E. Fogler, G. Poste, I. J. Fidler, *Cancer Res.* **1981**, *41*, 487.
- [321] F. Ahsan, I. P. Rivas, M. A. Khan, A. I. T. Suárez, *J. Controlled Release* **2002**, *79*, 29.
- [322] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, *Biomaterials* **2013**, *34*, 5628.
- [323] W. A. Jefferies, M. R. Brandon, S. V. Hunt, A. F. Williams, K. C. Gatter, D. Y. Mason, *Nature* **1984**, *312*, 162.
- [324] E. K. Park, S. B. Lee, Y. M. Lee, *Biomaterials* **2005**, *26*, 1053.
- [325] N. Parker, M. J. Turk, E. Westrick, J. D. Lewis, P. S. Low, C. P. Leamon, *Anal. Biochem.* **2005**, *338*, 284.
- [326] A. U. Buzdar, C. Marcus, G. R. Blumenschein, T. L. Smith, *Cancer* **1985**, *55*, 2761.
- [327] I. van Rooy, E. Mastrobattista, G. Storm, W. E. Hennink, R. M. Schiffelers, *J. Controlled Release* **2011**, *150*, 30.
- [328] R. H. Müller, K. Mäder, S. Gohla, *Eur. J. Pharm. Biopharm.* **2000**, *50*, 161.
- [329] I. P. Kaur, R. Bhandari, S. Bhandari, V. Kakkar, *J. Controlled Release* **2008**, *127*, 97.
- [330] K. Manjunath, V. Venkateswarlu, *J. Controlled Release* **2005**, *107*, 215.
- [331] B. Mishra, B. B. Patel, S. Tiwari, *Nanomed. Nanotechnol. Biol. Med.* **2010**, *6*, 9.
- [332] S. Jose, S. S. Anju, T. A. Cinu, N. A. Aleykutty, S. Thomas, E. B. Souto, *Int. J. Pharm.* **2014**, *474*, 6.
- [333] L. H. Reddy, R. K. Sharma, K. Chuttani, A. K. Mishra, R. R. Murthy, *AAPS J.* **2004**, *6*, 55.
- [334] P. R. Lockman, J. M. Koziara, R. J. Mumper, D. D. Allen, *J. Drug Targeting* **2004**, *12*, 635.
- [335] K. Letchford, H. Burt, *Eur. J. Pharm. Biopharm.* **2007**, *65*, 259.
- [336] G. S. Kwon, T. Okano, *Adv. Drug Delivery Rev.* **1996**, *21*, 107.
- [337] D. E. Discher, F. Ahmed, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323.
- [338] L. Liu, K. Guo, J. Lu, S. S. Venkatraman, D. Luo, K. C. Ng, E.-A. Ling, S. Moochhala, Y.-Y. Yang, *Biomaterials* **2008**, *29*, 1509.
- [339] L. Liu, S. S. Venkatraman, Y. Y. Yang, K. Guo, J. Lu, B. He, S. Moochhala, L. Kan, *Pept. Sci.* **2008**, *90*, 617.
- [340] K. Shao, R. Huang, J. Li, L. Han, L. Ye, J. Lou, C. Jiang, *J. Controlled Release* **2010**, *147*, 118.
- [341] M. Demeule, J. C. Currie, Y. Bertrand, C. Che, T. Nguyen, A. Regina, R. Gabathuler, J. P. Castaigne, R. Beliveau, *J. Neurochem.* **2008**, *106*, 1534.
- [342] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, *Biochemistry* **1991**, *30*, 5707.
- [343] R. S. Dhanikula, T. Hammady, P. Hildgen, *J. Pharm. Sci.* **2009**, *98*, 3748.
- [344] R. K. Tekade, T. Dutta, V. Gajbhiye, N. K. Jain, *J. Microencapsulation* **2009**, *26*, 287.
- [345] L. Albertazzi, L. Gherardini, M. Brondi, S. Sulis Sato, A. Bifone, T. Pizzorusso, G. M. Ratto, G. Bardi, *Mol. Pharm.* **2013**, *10*, 249.

- [346] W. Ke, K. Shao, R. Huang, L. Han, Y. Liu, J. Li, Y. Kuang, L. Ye, J. Lou, C. Jiang, *Biomaterials* **2009**, *30*, 6976.
- [347] R. Huang, W. Ke, L. Han, Y. Liu, K. Shao, L. Ye, J. Lou, C. Jiang, Y. Pei, *J. Cereb. Blood Flow Metab.* **2009**, *29*, 1914.
- [348] R.-Q. Huang, Y.-H. Qu, W.-L. Ke, J.-H. Zhu, Y.-Y. Pei, C. Jiang, *FASEB J.* **2007**, *21*, 1117.
- [349] M. Hasan, G. B. Messaoud, F. Michaux, A. Tamayol, C. J. F. Kahn, N. Belhaj, M. Linder, E. Arab-Tehrany, *RSC Adv.* **2016**, *6*, 45290.
- [350] S. A. Agnihotri, N. N. Mallikarjuna, T. M. Aminabhavi, *J. Controlled Release* **2004**, *100*, 5.
- [351] L. E. Gerweck, K. Seetharaman, *Cancer Res.* **1996**, *56*, 1194.
- [352] M. Kraus, B. Wolf, *Tumor Biol.* **1996**, *17*, 133.
- [353] S. Ruan, X. Cao, X. Cun, G. Hu, Y. Zhou, Y. Zhang, L. Lu, Q. He, H. Gao, *Biomaterials* **2015**, *60*, 100.
- [354] O. A. Andreev, A. D. Dupuy, M. Segala, S. Sandugu, D. A. Serra, C. O. Chichester, D. M. Engelman, Y. K. Reshetnyak, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7893.
- [355] S. Mura, J. Nicolas, P. Couvreur, *Nat. Mater.* **2013**, *12*, 991.
- [356] S. Nicol, *New Sci.* **1991**, *129*, 46.
- [357] M. Yemisci, Y. Gürsoy-Özdemir, S. Caban, E. Bodur, Y. Capan, T. Dalkara, *Methods Enzymol.* **2012**, *508*, 253.
- [358] S. Wang, T. Jiang, M. Ma, Y. Hu, J. Zhang, *Int. J. Pharm.* **2010**, *386*, 249.
- [359] M. Fazil, S. Md, S. Haque, M. Kumar, S. Baboota, J. kaur Sahni, J. Ali, *Eur. J. Pharm. Sci.* **2012**, *47*, 6.
- [360] X. Wang, N. Chi, X. Tang, *Eur. J. Pharm. Biopharm.* **2008**, *70*, 735.
- [361] T. Govender, S. Stolnik, M. C. Garnett, L. Illum, S. S. Davis, *J. Controlled Release* **1999**, *57*, 171.
- [362] M. T. Peracchia, R. Gref, Y. Minamitake, A. Domb, N. Lotan, R. Langer, *J. Controlled Release* **1997**, *46*, 223.
- [363] B. Semete, L. Booyens, Y. Lemmer, L. Kalombo, L. Katata, J. Verschoor, H. S. Swai, *Nanomed. Nanotechnol. Biol. Med.* **2010**, *6*, 662.
- [364] T. Verrecchia, G. Spenlehauer, D. V. Bazile, A. Murry-Brelier, Y. Archimbaud, M. Veillard, *J. Controlled Release* **1995**, *36*, 49.
- [365] M. Hadjidemetriou, K. Kostarelos, *Nat. Nano.* **2017**, *12*, 288.
- [366] J. Cheng, B. A. Teply, I. Sherifi, J. Sung, G. Luther, F. X. Gu, E. Levy-Nissenbaum, A. F. Radovic-Moreno, R. Langer, O. C. Farokhzad, *Biomaterials* **2007**, *28*, 869.
- [367] S. Gelperina, O. Maksimenko, A. Khalansky, L. Vanchugova, E. Shipulo, K. Abbasova, R. Berdiev, S. Wohlfart, N. Chepurnova, J. Kreuter, *Eur. J. Pharm. Biopharm.* **2010**, *74*, 157.
- [368] T. Hekmatara, S. Gelperina, V. Vogel, S.-R. Yang, J. Kreuter, *J. Nanosci. Nanotechnol.* **2009**, *9*, 5091.
- [369] R. N. Alyautdin, V. E. Petrov, K. Langer, A. Berthold, D. A. Kharkevich, J. Kreuter, *Pharm. Res.* **1997**, *14*, 325.
- [370] S. C. J. Steiniger, J. Kreuter, A. S. Khalansky, I. N. Skidan, A. I. Bobruskin, Z. S. Smirnova, S. E. Severin, R. Uhl, M. Kock, K. D. Geiger, S. E. Gelperina, *Int. J. Cancer* **2004**, *109*, 759.
- [371] B. Petri, A. Bootz, A. Khalansky, T. Hekmatara, R. Müller, R. Uhl, J. Kreuter, S. Gelperina, *J. Controlled Release* **2007**, *117*, 51.
- [372] J. Nicolas, P. Couvreur, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2009**, *1*, 111.

- [373] G. Yordanov, *Bulg. J. Chem.* **2012**, *1*, 61.
- [374] K. Gao, X. Jiang, *Int. J. Pharm.* **2006**, *310*, 213.
- [375] B. Stella, S. Arpicco, M. T. Peracchia, D. Desmaële, J. Hoebeke, M. Renoir, J. D'Angelo, L. Cattell, P. Couvreur, *J. Pharm. Sci.* **2000**, *89*, 1452.
- [376] U. Schroeder, P. Sommerfeld, B. A. Sabel, *Peptides* **1998**, *19*, 777.
- [377] A. Ambruosi, S. Gelperina, A. Khalansky, S. Tanski, A. Theisen, J. Kreuter, *J. Microencapsulation* **2006**, *23*, 582.
- [378] J. Kreuter, V. E. Petrov, D. A. Kharkevich, R. N. Alyautdin, *J. Controlled Release* **1997**, *49*, 81.
- [379] K. R. Chaudhari, M. Ukawala, A. S. Manjappa, A. Kumar, P. K. Mundada, A. K. Mishra, R. Mathur, J. Mönkkönen, R. S. R. Murthy, *Pharm. Res.* **2012**, *29*, 53.
- [380] K. Andrieux, P. Couvreur, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2009**, *1*, 463.
- [381] B. Wilson, M. K. Samanta, K. Santhi, K. P. S. Kumar, N. Paramakrishnan, B. Suresh, *Brain Res.* **2008**, *1200*, 159.
- [382] J. Kreuter, P. Ramge, V. Petrov, S. Hamm, S. E. Gelperina, B. Engelhardt, R. Alyautdin, H. Von Briesen, D. J. Begley, *Pharm. Res.* **2003**, *20*, 409.
- [383] D. Brambilla, J. Nicolas, B. Le Droumaguet, K. Andrieux, V. Marsaud, P.-O. Couraud, P. Couvreur, *Chem. Commun.* **2010**, *46*, 2602.
- [384] C.-Y. Huang, C.-M. Chen, Y.-D. Lee, *Int. J. Pharm.* **2007**, *338*, 267.
- [385] M. He, Z. Zhao, L. Yin, C. Tang, C. Yin, *Int. J. Pharm.* **2009**, *373*, 165.
- [386] X. Yang, M. Yang, B. Pang, M. Vara, Y. Xia, *Chem. Rev.* **2015**, *115*, 10410.
- [387] K. H. Song, C. Kim, C. M. Cobley, Y. Xia, L. V. Wang, *Nano Lett.* **2008**, *9*, 183.
- [388] S. J. Soenen, P. Rivera-Gil, J.-M. Montenegro, W. J. Parak, S. C. De Smedt, K. Braeckmans, *Nano Today* **2011**, *6*, 446.
- [389] A. Y. Mochizuki, I. M. Frost, M. B. Mastrodimos, A. S. Plant, A. C. Wang, T. B. Moore, R. M. Prins, P. S. Weiss, S. J. Jonas, *ACS Chem. Neurosci.* **2018**, *9*, 11.
- [390] G. Sonavane, K. Tomoda, K. Makino, *Colloids Surf. B* **2008**, *66*, 274.
- [391] S. K. Balasubramanian, J. Jittiwat, J. Manikandan, C.-N. Ong, E. Y. Liya, W.-Y. Ong, *Biomaterials* **2010**, *31*, 2034.
- [392] W. H. De Jong, W. I. Hagens, P. Krystek, M. C. Burger, A. J. A. M. Sips, R. E. Geertsma, *Biomaterials* **2008**, *29*, 1912.
- [393] C. Lasagna-Reeves, D. Gonzalez-Romero, M. A. Barria, I. Olmedo, A. Clos, V. M. S. Ramanujam, A. Urayama, L. Vergara, M. J. Kogan, C. Soto, *Biochem. Biophys. Res. Commun.* **2010**, *393*, 649.
- [394] R. Prades, S. Guerrero, E. Araya, C. Molina, E. Salas, E. Zurita, J. Selva, G. Egea, C. López-Iglesias, M. Teixidó, *Biomaterials* **2012**, *33*, 7194.
- [395] F. Sousa, S. Mandal, C. Garrovo, A. Astolfo, A. Bonifacio, D. Latawiec, R. H. Menk, F. Arfelli, S. Huewel, G. Legname, H.-J. Galla, S. Krol, *Nanoscale* **2010**, *2*, 2826.
- [396] G. Han, P. Ghosh, V. M. Rotello, *Nanomedicine* **2007**, *2*, 113.
- [397] F. Xu, W. Lu, H. Wu, L. Fan, X. Gao, X. Jiang, *J. Drug Targeting* **2009**, *17*, 423.
- [398] Y. Cheng, J. D. Meyers, R. S. Agnes, T. L. Doane, M. E. Kenney, A.-M. Broome, C. Burda, J. P. Basilion, *Small* **2011**, *7*, 2301.
- [399] E. Boisselier, D. Astruc, *Chem. Soc. Rev.* **2009**, *38*, 1759.
- [400] D. F. Ranney, H. H. Huffaker, *Ann. N. Y. Acad. Sci.* **1987**, *507*, 104.
- [401] B. Bonnemain, *J. Drug Targeting* **1998**, *6*, 167.
- [402] P. Xu, J. Li, B. Chen, X. Wang, X. Cai, H. Jiang, C. Wang, H. Zhang, *J. Biomed. Nanotechnol.* **2012**, *8*, 417.

- [403] S. P. Singh, M. F. Rahman, U. S. N. Murty, M. Mahboob, P. Grover, *Toxicol. Appl. Pharmacol.* **2013**, 266, 56.
- [404] M. Barrow, A. Taylor, P. Murray, M. J. Rosseinsky, D. J. Adams, *Chem. Soc. Rev.* **2015**, 44, 6733.
- [405] K. Hola, Z. Markova, G. Zoppellaro, J. Tucek, R. Zboril, *Biotechnol. Adv.* **2015**, 33, 1162.
- [406] S. J. H. Soenen, J. Baert, M. De Cuyper, *ChemBioChem* **2007**, 8, 2067.
- [407] B. Chertok, B. A. Moffat, A. E. David, F. Yu, C. Bergemann, B. D. Ross, V. C. Yang, *Biomaterials* **2008**, 29, 487.
- [408] A. S. Lübbe, C. Bergemann, W. Huhnt, T. Fricke, H. Riess, J. W. Brock, D. Huhn, *Cancer Res.* **1996**, 56, 4694.
- [409] A. S. Lübbe, C. Bergemann, H. Riess, F. Schriever, P. Reichardt, K. Possinger, M. Matthias, B. Dörken, F. Herrmann, R. Gürtler, *Cancer Res.* **1996**, 56, 4686.
- [410] O. Shimoni, B. Shi, P. A. Adlard, A. I. Bush, *J. Mol. Neurosci.* **2016**, 60, 405.
- [411] W. W.-W. Hsiao, Y. Y. Hui, P.-C. Tsai, H.-C. Chang, *Acc. Chem. Res.* **2016**, 49, 400.
- [412] D. Ni, J. Zhang, W. Bu, H. Xing, F. Han, Q. Xiao, Z. Yao, F. Chen, Q. He, J. Liu, *ACS Nano* **2014**, 8, 1231.
- [413] H. Xing, X. Zheng, Q. Ren, W. Bu, W. Ge, Q. Xiao, S. Zhang, C. Wei, H. Qu, Z. Wang, *Sci. Rep.* **2013**, 3, 1751.
- [414] A. E. Gulyaev, S. E. Gelperina, I. N. Skidan, A. S. Antropov, G. Y. Kivman, J. Kreuter, *Pharm. Res.* **1999**, 16, 1564.
- [415] P. R. Lockman, R. J. Mumper, M. A. Khan, D. D. Allen, *Drug Dev. Ind. Pharm.* **2002**, 28, 1.
- [416] Q. Dai, N. Bertleff-Zieschang, J. A. Braunger, M. Björnmalm, C. Cortez-Jugo, F. Caruso, *Adv. Healthcare Mater.* **2018**, 7, 1700575.
- [417] W. Liang, J. K. W. Lam, in *Molecular Regulation of Endocytosis*, (Ed: B. Ceresa), InTech, Rijeka, Croatia **2012**, Ch. 17.
- [418] L. Rey, J. C. May, *Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products*, CRC Press, London, UK **2010**.
- [419] M. P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. Baldelli Bombelli, K. A. Dawson, *J. Am. Chem. Soc.* **2011**, 133, 2525.
- [420] M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, K. A. Dawson, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, 105, 14265.
- [421] I. Lynch, K. A. Dawson, *Nano Today* **2008**, 3, 40.
- [422] I. Lynch, T. Cedervall, M. Lundqvist, C. Cabaleiro-Lago, S. Linse, K. A. Dawson, *Adv. Colloid Interface Sci.* **2007**, 134, 167.
- [423] D. E. Owens, N. A. Peppas, *Int. J. Pharm.* **2006**, 307, 93.
- [424] H. Hillaireau, P. Couvreur, *Cell. Mol. Life Sci.* **2009**, 66, 2873.
- [425] M. I. Papisov, A. Bogdanov, B. Schaffer, N. Nossiff, T. Shen, R. Weissleder, T. J. Brady, *J. Magn. Magn. Mater.* **1993**, 122, 383.
- [426] B. Chertok, A. E. David, V. C. Yang, *Biomaterials* **2010**, 31, 6317.
- [427] L. Grislain, P. Couvreur, V. Lenaerts, M. Roland, D. Deprez-Decampeneere, P. Speiser, *Int. J. Pharm.* **1983**, 15, 335.
- [428] S. M. Moghimi, A. C. Hunter, J. C. Murray, *Pharmacol. Rev.* **2001**, 53, 283.
- [429] M. A. Dobrovolskaia, S. E. McNeil, *Nat. Nano.* **2007**, 2, 469.
- [430] F. F. Davis, *Adv. Drug Delivery Rev.* **2002**, 54, 457.
- [431] J.-M. Rabanel, P. Hildgen, X. Banquy, *J. Controlled Release* **2014**, 185, 71.
- [432] G. Ruan, S.-S. Feng, *Biomaterials* **2003**, 24, 5037.

- [433] J. L. Perry, K. G. Reuter, M. P. Kai, K. P. Herlihy, S. W. Jones, J. C. Luft, M. Napier, J. E. Bear, J. M. DeSimone, *Nano Lett.* **2012**, *12*, 5304.
- [434] S. I. Jeon, J. H. Lee, J. D. Andrade, P. G. De Gennes, *J. Colloid Interface Sci.* **1991**, *142*, 149.
- [435] M. Vittaz, D. Bazile, G. Spenlehauer, T. Verrecchia, M. Veillard, F. Puisieux, D. Labarre, *Biomaterials* **1996**, *17*, 1575.
- [436] R. Gref, M. Lück, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R. H. Müller, *Colloids Surf. B* **2000**, *18*, 301.
- [437] J. K. Gbadamosi, A. C. Hunter, S. M. Moghimi, *FEBS Lett.* **2002**, *532*, 338.
- [438] W. G. Kreyling, A. M. Abdelmonem, Z. Ali, F. Alves, M. Geiser, N. Haberl, R. Hartmann, S. Hirn, D. J. de Aberasturi, K. Kantner, G. Khadem-Saba, J.-M. Montenegro, J. Rejman, T. Rojo, I. R. de Larramendi, R. Ufartes, A. Wenk, W. J. Parak, *Nat. Nano.* **2015**, *10*, 619.
- [439] M. J. Webber, E. A. Appel, B. Vinciguerra, A. B. Cortinas, L. S. Thapa, S. Jhunjhunwala, L. Isaacs, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 14189.
- [440] A. P. Chapman, *Adv. Drug Delivery Rev.* **2002**, *54*, 531.
- [441] C. J. Hukshorn, F. M. H. van Dielen, W. A. Buurman, M. S. Westerterp-Plantenga, L. A. Campfield, W. H. M. Saris, *Int. J. Obes.* **2002**, *26*, 504.
- [442] W. M. Pardridge, D. Wu, T. Sakane, *Pharm. Res.* **1998**, *15*, 576.
- [443] K. Yoshida, G. F. Burton, J. S. McKinney, H. Young, E. F. Ellis, *Stroke* **1992**, *23*, 865.
- [444] K. Maruyama, T. Takizawa, T. Yuda, S. J. Kennel, L. Huang, M. Iwatsuru, *Biochim. Biophys. Acta Biomembr.* **1995**, *1234*, 74.
- [445] T. M. Allen, E. Brandeis, C. B. Hansen, G. Y. Kao, S. Zalipsky, *Biochim. Biophys. Acta Biomembr.* **1995**, *1237*, 99.
- [446] J. J. F. Verhoef, J. F. Carpenter, T. J. Anchordoquy, H. Schellekens, *Drug Discovery Today* **2014**, *19*, 1945.
- [447] K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, *Angew. Chem.* **2010**, *122*, 6430; *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 6288.
- [448] K. Środa, J. Rydlewski, M. Langner, A. Kozubek, M. Grzybek, A. F. Sikorski, *Cell. Mol. Biol. Lett.* **2005**, *10*, 37.
- [449] P. Calvo, B. Gouritin, H. Chacun, D. Desmaële, J. D'Angelo, J.-P. Noel, D. Georjgin, E. Fattal, J. P. Andreux, P. Couvreur, *Pharm. Res.* **2001**, *18*, 1157.
- [450] L. Illum, S. S. Davis, R. H. Müller, E. Mak, P. West, *Life Sci.* **1987**, *40*, 367.
- [451] R. Müller, S. Maaben, H. Weyhers, W. Mehnert, *J. Drug Targeting* **1996**, *4*, 161.
- [452] S. M. Moghimi, I. S. Muir, L. Illum, S. S. Davis, V. Kolb-Bachofen, *Biochim. Biophys. Acta Mol. Cell Res.* **1993**, *1179*, 157.
- [453] L. Illum, P. West, C. Washington, S. S. Davis, *Int. J. Pharm.* **1989**, *54*, 41.
- [454] O. Al-Hanbali, K. J. Rutt, D. K. Sarker, A. C. Hunter, S. M. Moghimi, *J. Nanosci. Nanotechnol.* **2006**, *6*, 3126.
- [455] J.-C. Olivier, L. Fenart, R. Chauvet, C. Pariat, R. Cecchelli, W. Couet, *Pharm. Res.* **1999**, *16*, 1836.
- [456] G. K. Such, Y. Yan, A. P. R. Johnston, S. T. Gunawan, F. Caruso, *Adv. Mater.* **2015**, *27*, 2278.
- [457] G. Raab, M. Klagsbrun, *Biochim. Biophys. Acta Rev. Cancer* **1997**, *1333*, F179.
- [458] K. Ulbrich, T. Hekmatara, E. Herbert, J. Kreuter, *Eur. J. Pharm. Biopharm.* **2009**, *71*, 251.

- [459] H. J. Lee, B. Engelhardt, J. Lesley, U. Bickel, W. M. Pardridge, *J. Pharmacol. Exp. Ther.* **2000**, 292, 1048.
- [460] M. Malhotra, C. Tomaro-Duchesneau, S. Saha, S. Prakash, *Int. J. Biomater.* **2013**, 2013, 1.
- [461] M. J. Coloma, H. J. Lee, A. Kurihara, E. M. Landaw, R. J. Boado, S. L. Morrison, W. M. Pardridge, *Pharm. Res.* **2000**, 17, 266.
- [462] R. J. Boado, Y. Zhang, Y. Zhang, W. M. Pardridge, *Biotechnol. Bioeng.* **2007**, 96, 381.
- [463] K. Michaelis, M. M. Hoffmann, S. Dreis, E. Herbert, R. N. Alyautdin, M. Michaelis, J. Kreuter, K. Langer, *J. Pharmacol. Exp. Ther.* **2006**, 317, 1246.
- [464] W. Pan, A. J. Kastin, T. C. Zankel, P. van Kerkhof, T. Terasaki, G. Bu, *J. Cell Sci.* **2004**, 117, 5071.
- [465] P. Wang, Y. Xue, X. Shang, Y. Liu, *Cell. Mol. Neurobiol.* **2010**, 30, 717.
- [466] E. Malito, B. Bursulaya, C. Chen, P. L. Surdo, M. Picchianti, E. Balducci, M. Biancucci, A. Brock, F. Berti, M. J. Bottomley, *Proc. Natl. Acad. Sci. U. S. A.* **2012**, 109, 5229.
- [467] D. Wu, W. M. Pardridge, *Pharm. Res.* **1999**, 16, 415.
- [468] A. Muruganandam, J. Tanha, S. Narang, D. Stanimirovic, *FASEB J.* **2002**, 16, 240.
- [469] G. K. Farrington, N. Caram-Salas, A. S. Haqqani, E. Brunette, J. Eldredge, B. Pepinsky, G. Antognetti, E. Baumann, W. Ding, E. Garber, *FASEB J.* **2014**, 28, 4764.
- [470] K. S. Rao, M. K. Reddy, J. L. Horning, V. Labhasetwar, *Biomaterials* **2008**, 29, 4429.
- [471] W. Lu, Y.-Z. Tan, K.-L. Hu, X.-G. Jiang, *Int. J. Pharm.* **2005**, 295, 247.
- [472] W. Lu, Y. Zhang, Y.-Z. Tan, K.-L. Hu, X.-G. Jiang, S.-K. Fu, *J. Controlled Release* **2005**, 107, 428.
- [473] Q. Dai, Y. Yan, J. Guo, M. Björnmalm, J. Cui, H. Sun, F. Caruso, *ACS Macro Lett.* **2015**, 4, 1259.
- [474] A. Béduneau, P. Saulnier, J.-P. Benoit, *Biomaterials* **2007**, 28, 4947.
- [475] C. B. Howard, N. Fletcher, Z. H. Houston, A. V. Fuchs, N. R. B. Boase, J. D. Simpson, L. J. Raftery, T. Ruder, M. L. Jones, C. J. de Bakker, S. M. Mahler, K. J. Thurecht, *Adv. Healthcare Mater.* **2016**, 5, 2055.
- [476] C. Saraiva, C. Praça, R. Ferreira, T. Santos, L. Ferreira, L. Bernardino, *J. Controlled Release* **2016**, 235, 34.
- [477] M. Colombo, L. Fiandra, G. Alessio, S. Mazzucchelli, M. Nebuloni, C. De Palma, K. Kantner, B. Pelaz, R. Rotem, F. Corsi, W. J. Parak, D. Prospero, *Nat. Commun.* **2016**, 7, 13818.
- [478] D. T. Wiley, P. Webster, A. Gale, M. E. Davis, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, 110, 8662.
- [479] C. H. J. Choi, C. A. Alabi, P. Webster, M. E. Davis, *Proc. Natl. Acad. Sci. U. S. A.* **2010**, 107, 1235.
- [480] F. J. Martinez-Veracochea, D. Frenkel, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, 108, 10963.
- [481] P. Ponka, C. N. Lok, *Int. J. Biochem. Cell Biol.* **1999**, 31, 1111.
- [482] W. M. Pardridge, J. L. Buciak, P. M. Friden, *J. Pharmacol. Exp. Ther.* **1991**, 259, 66.
- [483] L. Plum, M. Schubert, J. C. Brüning, *Trends Endocrinol. Metab.* **2005**, 16, 59.
- [484] J. C. Olivier, L. Fenart, R. Chauvet, C. Pariat, R. Cecchelli, W. Couet, *Pharm. Res.* **1999**, 16, 1836.
- [485] U. Hülsermann, M. M. Hoffmann, U. Massing, G. Fricker, *J. Drug Targeting* **2009**, 17, 610.

- [486] A. Zensi, D. Begley, C. Pontikis, C. Legros, L. Mihoreanu, S. Wagner, C. Büchel, H. von Briesen, J. Kreuter, *J. Controlled Release* **2009**, *137*, 78.
- [487] B. Ji, J. Maeda, M. Higuchi, K. Inoue, H. Akita, H. Harashima, T. Suhara, *Life Sci.* **2006**, *78*, 851.
- [488] J. Lalani, Y. Raichandani, R. Mathur, M. Lalan, K. Chutani, A. K. Mishra, A. Misra, *J. Biomed. Nanotechnol.* **2012**, *8*, 918.
- [489] M. Demeule, J. Poirier, J. Jodoin, Y. Bertrand, R. R. Desrosiers, C. Dagenais, T. Nguyen, J. Lanthier, R. Gabathuler, M. Kennard, W. A. Jefferies, D. Karkan, S. Tsai, L. Fenart, R. Cecchelli, R. Béliveau, *J. Neurochem.* **2002**, *83*, 924.
- [490] I. K. U. Moroo, M. Ujiie, B. L. Walker, J. W. C. Tiong, T. Z. Vitalis, D. Karkan, R. Gabathuler, A. R. Moise, W. A. Jefferies, *Microcirculation* **2003**, *10*, 457.
- [491] R. Gabathuler, G. Arthur, M. Kennard, Q. Chen, S. Tsai, J. Yang, W. Schoorl, T. Z. Vitalis, W. A. Jefferies, *Int. Congr. Ser.* **2005**, *1277*, 171.
- [492] G. Bu, H. J. Geuze, G. J. Strous, A. L. Schwartz, *EMBO J.* **1995**, *14*, 2269.
- [493] I. Warshawsky, G. Bu, A. L. Schwartz, *J. Clin. Invest.* **1993**, *92*, 937.
- [494] G. Giannini, R. Rappuoli, G. Ratti, *Nucleic Acids Res.* **1984**, *12*, 4063.
- [495] J. F. Ross, P. K. Chaudhuri, M. Ratnam, *Cancer* **1994**, *73*, 2432.
- [496] H. S. Yoo, T. G. Park, *J. Controlled Release* **2004**, *96*, 273.
- [497] P. Calvo, B. Gouritin, H. Villarroya, F. Eclancher, C. Giannavola, C. Klein, J. P. Andreux, P. Couvreur, *Eur. J. Neurosci.* **2002**, *15*, 1317.
- [498] S. Şimşek, H. Eroğlu, B. Kurum, K. Ulubayram, *J. Microencapsulation* **2013**, *30*, 10.
- [499] Y.-J. Wang, M. Larsson, W.-T. Huang, S.-H. Chiou, S. J. Nicholls, J.-I. Chao, D.-M. Liu, *Prog. Polym. Sci.* **2016**, *57*, 153.
- [500] A. Mishra, G. H. Lai, N. W. Schmidt, V. Z. Sun, A. R. Rodriguez, R. Tong, L. Tang, J. Cheng, T. J. Deming, D. T. Kamei, G. C. L. Wong, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 16883.
- [501] S. Deshayes, M. C. Morris, G. Divita, F. Heitz, *Cell. Mol. Life Sci.* **2005**, *62*, 1839.
- [502] M. Lindgren, M. Hällbrink, A. Prochiantz, Ü. Langel, *Trends Pharmacol. Sci.* **2000**, *21*, 99.
- [503] E. Koren, V. P. Torchilin, *Trends Mol. Med.* **2012**, *18*, 385.
- [504] A. K. Cardozo, V. Buchillier, M. Mathieu, J. Chen, F. Ortis, L. Ladrière, N. Allaman-Pillet, O. Poirot, S. Kellenberger, J. S. Beckmann, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 2222.
- [505] J. J. Richardson, J. Cui, M. Björnmalm, J. A. Braunger, H. Ejima, F. Caruso, *Chem. Rev.* **2016**, *116*, 14828.
- [506] J. F. Poduslo, G. L. Curran, *J. Neurochem.* **1996**, *66*, 1599.
- [507] A. R. Bender, H. von Briesen, J. Kreuter, I. B. Duncan, H. Rübsamen-Waigmann, *Antimicrob. Agents Chemother.* **1996**, *40*, 1467.
- [508] E. Afergan, H. Epstein, R. Dahan, N. Koroukhov, K. Rohekar, H. D. Danenberg, G. Golomb, *J. Controlled Release* **2008**, *132*, 84.
- [509] J. A. Champion, S. Mitragotri, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 4930.
- [510] J. A. Champion, S. Mitragotri, *Pharm. Res.* **2009**, *26*, 244.
- [511] Y. A. N. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko, D. E. Discher, *Nat. Nano.* **2007**, *2*, 249.
- [512] G. Sharma, D. T. Valenta, Y. Altman, S. Harvey, H. Xie, S. Mitragotri, J. W. Smith, *J. Controlled Release* **2010**, *147*, 408.
- [513] S. E. A. Gratton, P. A. Ropp, P. D. Pohlhaus, J. C. Luft, V. J. Madden, M. E. Napier, J. M. DeSimone, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11613.

- [514] J. A. Braunger, M. Björnmalm, N. A. Isles, J. Cui, T. M. A. Henderson, A. J. O'Connor, F. Caruso, *Biomater. Sci.* **2017**, *5*, 267.
- [515] M. Björnmalm, M. Faria, X. Chen, J. Cui, F. Caruso, *Langmuir* **2016**, *32*, 10995.
- [516] A. C. Anselmo, M. Zhang, S. Kumar, D. R. Vogus, S. Menegatti, M. E. Helgeson, S. Mitragotri, *ACS Nano* **2015**, *9*, 3169.
- [517] A. C. Anselmo, S. Mitragotri, *Adv. Drug Deliv. Rev.* **2017**, *108*, 51.
- [518] A. Albanese, P. S. Tang, W. C. W. Chan, *Annu. Rev. Biomed. Eng.* **2012**, *14*, 1.
- [519] Y. Yan, M. Björnmalm, F. Caruso, *Chem. Mater.* **2014**, *26*, 452.
- [520] R. A. Petros, J. M. DeSimone, *Nat. Rev. Drug Discovery* **2010**, *9*, 615.
- [521] Y. Zhao, M. J. Haney, N. L. Klyachko, S. Li, S. L. Booth, S. M. Higginbotham, J. Jones, M. C. Zimmerman, R. L. Mosley, A. V. Kabanov, *Nanomedicine* **2011**, *6*, 25.
- [522] B. Zarabi, A. Nan, J. Zhuo, R. Gullapalli, H. Ghandehari, *Mol. Pharm.* **2006**, *3*, 550.
- [523] R. M. Senior, H. D. Gresham, G. L. Griffin, E. J. Brown, A. E. Chung, *J. Clin. Invest.* **1992**, *90*, 2251.
- [524] X. Jiang, H. Gao, *Neurotoxicity of Nanomaterials and Nanomedicine*, Academic Press, Cambridge, UK **2016**.
- [525] Y. H. Bae, K. Park, *J. Controlled Release* **2011**, *153*, 198.
- [526] W. M. Pardridge, *Brain Drug Targeting: The Future of Brain Drug Development*, Cambridge University Press, Cambridge, UK **2001**.
- [527] C. M. Peppiatt, C. Howarth, P. Mobbs, D. Attwell, *Nature* **2006**, *443*, 700.
- [528] R. K. Jain, T. Stylianopoulos, *Nat. Rev. Clin. Oncol.* **2010**, *7*, 653.
- [529] T. Stylianopoulos, R. K. Jain, *Nanomed. Nanotechnol. Biol. Med.* **2015**, *11*, 1893.
- [530] F. Danhier, *J. Controlled Release* **2016**, *244*, 108.
- [531] J. Folkman, *Semin. Oncol.* **2002**, *29*, 15.
- [532] A. K. Iyer, G. Khaled, J. Fang, H. Maeda, *Drug Discovery Today* **2006**, *11*, 812.
- [533] F.-R. E. Curry, *Nat. Nano.* **2016**, *11*, 494.
- [534] Y. Matsumoto, J. W. Nichols, K. Toh, T. Nomoto, H. Cabral, Y. Miura, R. J. Christie, N. Yamada, T. Ogura, M. R. Kano, Y. Matsumura, N. Nishiyama, T. Yamasoba, Y. H. Bae, K. Kataoka, *Nat. Nano.* **2016**, *11*, 533.
- [535] M. K. Reddy, V. Labhasetwar, *FASEB J.* **2009**, *23*, 1384.
- [536] S. Kannan, H. Dai, R. S. Navath, B. Balakrishnan, A. Jyoti, J. Janisse, R. Romero, R. M. Kannan, *Sci. Transl. Med.* **2012**, *4*, 130ra46.
- [537] I. F. Tannock, D. Rotin, *Cancer Res.* **1989**, *49*, 4373.
- [538] J. W. Nichols, Y. H. Bae, *J. Controlled Release* **2014**, *190*, 451.
- [539] P. Galvin, D. Thompson, K. B. Ryan, A. McCarthy, A. C. Moore, C. S. Burke, M. Dyson, B. D. MacCraith, Y. K. Gun'ko, M. T. Byrne, Y. Volkov, C. Keely, E. Keehan, M. Howe, C. Duffy, R. MacLoughlin, *Cell. Mol. Life Sci.* **2012**, *69*, 389.
- [540] J. Cui, J. J. Richardson, M. Björnmalm, M. Faria, F. Caruso, *Acc. Chem. Res.* **2016**, *49*, 1139.
- [541] J.-Z. Du, X.-J. Du, C.-Q. Mao, J. Wang, *J. Am. Chem. Soc.* **2011**, *133*, 17560.
- [542] A. B. Hjelmeland, Q. Wu, J. M. Heddleston, G. S. Choudhary, J. MacSwords, J. D. Lathia, R. McLendon, D. Lindner, A. Sloan, J. N. Rich, *Cell Death Differ.* **2011**, *18*, 829.
- [543] C. S. Atwood, R. D. Moir, X. Huang, R. C. Scarpa, N. M. E. Bacarra, D. M. Romano, M. A. Hartshorn, R. E. Tanzi, A. I. Bush, *J. Biol. Chem.* **1998**, *273*, 12817.
- [544] W. A. C. Mutch, A. J. Hansen, *J. Cereb. Blood Flow Metab.* **1984**, *4*, 17.
- [545] J. R. Duguid, C. W. Bohmont, N. G. Liu, W. W. Tourtellotte, *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 7260.

- [546] P. Pradhan, J. Giri, F. Rieken, C. Koch, O. Mykhaylyk, M. Döblinger, R. Banerjee, D. Bahadur, C. Plank, *J. Controlled Release* **2010**, *142*, 108.
- [547] L. Jiang, Q. Zhou, K. Mu, H. Xie, Y. Zhu, W. Zhu, Y. Zhao, H. Xu, X. Yang, *Biomaterials* **2013**, *34*, 7418.
- [548] R. Busto, W. D. Dietrich, M. Y. T. Globus, M. D. Ginsberg, *Stroke* **1989**, *20*, 1113.
- [549] R. Busto, W. D. Dietrich, M. Y. T. Globus, I. Valdés, P. Scheinberg, M. D. Ginsberg, *J. Cereb. Blood Flow Metab.* **1987**, *7*, 729.
- [550] N. Hayashi, T. Hirayama, A. Udagawa, W. Daimon, M. Ohata, in *Brain Edema IX: Proceedings of the Ninth International Symposium Tokyo, May 16–19, 1993*, (Eds: U. Ito, A. Baethmann, K.-A. Hossmann, T. Kuroiwa, A. Marmarou, H.-J. Reulen, K. Takakura), Springer, Vienna, Italy **1994**, 541.
- [551] T. Noyhouzer, C. L’Homme, I. Beaulieu, S. Mazurkiewicz, S. Kuss, H.-B. Kraatz, S. Canesi, J. Mauzeroll, *Langmuir* **2016**, *32*, 4169.
- [552] M. Y. Aksenov, W. R. Markesbery, *Neurosci. Lett.* **2001**, *302*, 141.
- [553] M. Bergeron, S. M. Evans, F. R. Sharp, C. J. Koch, E. M. Lord, D. M. Ferriero, *Neuroscience* **1999**, *89*, 1357.
- [554] L.-P. Liang, M. Patel, *Free Radical Biol. Med.* **2006**, *40*, 316.
- [555] C. Zhang, X. Wan, X. Zheng, X. Shao, Q. Liu, Q. Zhang, Y. Qian, *Biomaterials* **2014**, *35*, 456.
- [556] Y. Li, H. He, X. Jia, W.-L. Lu, J. Lou, Y. Wei, *Biomaterials* **2012**, *33*, 3899.
- [557] H. Xin, X. Jiang, J. Gu, X. Sha, L. Chen, K. Law, Y. Chen, X. Wang, Y. Jiang, X. Fang, *Biomaterials* **2011**, *32*, 4293.
- [558] H. Xin, X. Sha, X. Jiang, W. Zhang, L. Chen, X. Fang, *Biomaterials* **2012**, *33*, 8167.
- [559] Q. Sun, T. Ojha, F. Kiessling, T. Lammers, Y. Shi, *Biomacromolecules* **2017**, *18*, 1449.
- [560] O. Veisoh, C. Sun, C. Fang, N. Bhattarai, J. Gunn, F. Kievit, K. Du, B. Pullar, D. Lee, R. G. Ellenbogen, J. Olson, M. Zhang, *Cancer Res.* **2009**, *69*, 6200.
- [561] K. N. Sugahara, T. Teesalu, P. P. Karmali, V. R. Kotamraju, L. Agemy, O. M. Girard, D. Hanahan, R. F. Mattrey, E. Ruoslahti, *Cancer Cell* **2009**, *16*, 510.
- [562] K. N. Sugahara, T. Teesalu, P. P. Karmali, V. R. Kotamraju, L. Agemy, D. R. Greenwald, E. Ruoslahti, *Science* **2010**, *328*, 1031.
- [563] X. Qian, X.-H. Peng, D. O. Ansari, Q. Yin-Goen, G. Z. Chen, D. M. Shin, L. Yang, A. N. Young, M. D. Wang, S. Nie, *Nat. Biotechnol.* **2008**, *26*, 83.
- [564] K. F. Pirollo, E. H. Chang, *Trends Biotechnol.* **2008**, *26*, 552.
- [565] D. B. Kirpotin, D. C. Drummond, Y. Shao, M. R. Shalaby, K. Hong, U. B. Nielsen, J. D. Marks, C. C. Benz, J. W. Park, *Cancer Res.* **2006**, *66*, 6732.
- [566] A. M. Wu, P. J. Yazaki, S.-W. Tsai, K. Nguyen, A.-L. Anderson, D. W. McCarthy, M. J. Welch, J. E. Shively, L. E. Williams, A. A. Raubitschek, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 8495.
- [567] M. Torrice, *ACS Cent. Sci.* **2016**, *2*, 434.
- [568] J. K. Atwal, Y. Chen, C. Chiu, D. L. Mortensen, W. J. Meilandt, Y. Liu, C. E. Heise, K. Hoyte, W. Luk, Y. Lu, *Sci. Transl. Med.* **2011**, *3*, 84ra43.
- [569] S. M. Paul, *Sci. Transl. Med.* **2011**, *3*, 84ps20.
- [570] F. Hefti, *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 239.
- [571] Y. Zhang, W. M. Pardridge, *Brain Res.* **2001**, *889*, 49.
- [572] A. Minn, J.-F. Gherzi-Egea, R. Perrin, B. Leininger, G. Siest, *Brain Res. Rev.* **1991**, *16*, 65.
- [573] D. Maysinger, M. Behrendt, M. Lalancette-Hébert, J. Kriz, *Nano Lett.* **2007**, *7*, 2513.

- [574] F. Barandeh, P.-L. Nguyen, R. Kumar, G. J. Iacobucci, M. L. Kuznicki, A. Kosterman, E. J. Bergey, P. N. Prasad, S. Gunawardena, *PLoS One* **2012**, 7, e29424.
- [575] R. Kontermann, *mAbs* **2012**, 4, 182.
- [576] L. M. Maness, W. A. Banks, J. E. Zadina, A. J. Kastin, *Brain Res.* **1995**, 700, 83.
- [577] A. Cavalcanti, B. Shirinzadeh, T. Fukuda, S. Ikeda, *Int. J. Robotics Res.* **2009**, 28, 558.

Figure 1. Graphical overview of the process leading to the successful, nanomaterial-mediated treatment of neurological diseases. Parts of this figure are adapted with permission.^[12]

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Figure 2. Barriers of the CNS. The three main barriers of the CNS include (a) the arachnoid barrier, (b) the blood–brain barrier (BBB), and (c) the blood–cerebrospinal fluid barrier (BCSFB), all of which are identifiable in (d), a schematic coronal brain section. Of the three barriers, the BBB maintains closest proximity to the brain parenchyma, and therefore offers the most viable opportunity for CNS drug delivery. Adapted with permission.^[25] Copyright 2017, Springer Nature.

Figure 3. Schematic illustration of the structure of the neurovascular unit (NVU). The NVU comprises various cell types, including neurons, BMECs, vascular smooth muscle cells (VSMCs), astrocytes, microglia, pericytes, oligodendrocytes, mast cells and even circulating leukocytes. VSMCs surround larger blood vessels and play a major role in controlling CBF, but are gradually replaced by pericytes as the vessels narrow to form much smaller capillaries. These capillaries or microvessels demonstrate closest proximity to the brain parenchymal tissues. Precise, regulated cross-talk occurs between the cells of the NVU to ultimately determine the overall phenotype of the BBB and ensure that homeostatic equilibrium is maintained. Biological dimension data sourced from refs [6, 35-39].

Figure 4. Physical barrier of the BBB. The low permeability of the BBB is primarily derived from its physical barrier, which comprises tight junction proteins, adherens junction proteins, apico-basal polarity and a luminal surface-bound glycocalyx. Reproduced with permission.^[2]

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Figure 5. Carrier transport mechanisms at the BBB. Several different carriers facilitate blood-to-brain movement and brain-to-blood movement of key nutrient molecules, thereby maintaining homeostatic equilibrium of the CNS. Reproduced with permission.^[51] Copyright 2012, Wolters Kluwer Health.

Figure 6. Schematic illustration of the healthy brain. The immune system is relatively quiescent under normal physiological conditions, with microglia and T cells predominantly responsible for CNS immune surveillance. T cells enter the CSF from the choroid plexus (CP). Adapted with permission.^[63] Copyright 2016, Elsevier.

Figure 7. Progressive changes to the blood–brain barrier (BBB) and the neurovascular unit (NVU) under pathological conditions. Various disease-specific triggers can initiate CNS pathology, including genetic susceptibilities (e.g., predisposing gene variants), environmental factors (e.g., low vitamin D, exposure to heavy metals, prolonged smoke inhalation), vascular factors (e.g., ischemia), and infective agents (e.g., bacteria, viruses, fungal pathogens). Individually, these factors are unlikely to cause significant and lasting CNS impairment. However, their coalescence can cause a cascade of pathological events leading to dysregulation of NVU cells and defective signaling, breakdown of BBB integrity and permeability, immune cell activation, and ultimately neuronal injury, inflammation and neurodegeneration. Many cells that normally maintain CNS homeostasis—including pericytes, astrocytes, microglia and BMECs—become dysregulated and take on a distressed phenotype. They begin to aberrantly release various inflammatory compounds, including cytokines (e.g., IL-1 β , IL-6, TNF- α), lipid mediators (e.g., prostaglandins), oxidative compounds (e.g., ROS, free radical nitrogen oxide (NO)), vasogenic agents (e.g., histamine, vascular endothelial growth factor (VEGF)), and enzymes (e.g., matrix metalloproteinases

(MMPs), nitric oxide synthase (NOS), tissue plasminogen activator (tPa)). These compounds act to promote breakdown of the various NVU components, further propagating the disease process and inevitably leading to a worsening of clinical symptoms.

Figure 8. Immune cell trafficking across BBB during CNS pathology. Increased leukocyte trafficking and increased release of inflammatory mediators are considered clear indications of neuroinflammation. Adapted with permission.^[73] Copyright 2011, Springer Nature.

Figure 9. Inflammatory mediators released by peripheral immune cells in CNS pathology. These include cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs), which act on the cells of the NVU to cause BBB disruption and neuroinflammation. Reproduced with permission.^[77] Copyright 2011, Wiley.

Figure 10. Illustration of the primary hallmarks of AD, including: (i) the build-up of A β peptide, and (ii) the accumulation of hyperphosphorylated, microtubule-associated tau protein, resulting in intraneuronal neurofibrillary tangles. Reproduced with permission.^[86] Copyright 2011, American Association for the Advancement of Science.

Figure 11. Schematic illustration of the pathophysiological processes underlying dysregulation of the NVU and development of the MS phenotype. Reproduced with permission.^[115] Copyright 2012, Springer Nature.

Figure 12. Schematic illustration of the NVU under PD conditions. Hallmarks of the pathology include intraneuronal alpha-synuclein accumulation, mitochondrial dysfunction, and neuronal cell death. Reproduced under the terms of the CC-BY 3.0 license.^[125] Copyright

2014, Frontiers Media SA.

Figure 13. Schematic overview of the pathways that can lead to stroke, and the regions of the brain that can be affected as a result. Adapted with permission.^[131] Copyright 2014, Nature Publishing Group.

Figure 14. Schematic illustration of the NVU after an ischemic stroke event. MMPs, plasminogen activators and other proteases are upregulated, and these act on cells of the NVU to cause widespread BBB disruption. Stroke-induced brain injury is exacerbated by the subsequent infiltration of inflammatory cells through the damaged BBB into the parenchymal space. Reproduced with permission.^[142] Copyright 2003, Springer Nature.

Figure 15. Summary of drug delivery methods that bypass the BBB, including (A) intracerebroventricular (ICV), (B) convection-enhanced delivery (CED), (C) intraparenchymal/intracerebral, (D) intrathecal, (E) intranasal and (F) intratympanic routes of administration.

Figure 16. Comparison between locoregional drug distribution profiles for CED versus intracerebral administration. Results show that greater drug dispersion from the site of injection occurs for CED, due to convective fluid movement as well as diffusion. Reproduced with permission.^[153] Copyright 2013, Elsevier.

Figure 17. Noninvasive CNS drug delivery strategies that exploit endogenous pathways across the BBB. Three such pathways include paracellular transport, passive transcellular diffusion, and carrier-mediated transport.

Figure 18. Noninvasive CNS drug delivery strategies that exploit endogenous pathways across the BBB (continued). Three other pathways include adsorptive-mediated transcytosis (AMT), receptor-mediated transcytosis (RMT) and cell-mediated transport. Drug molecules and NPs can be specifically functionalized to exploit these mechanisms of transport across the BBB.

Figure 19. Different modes of BBB internalization. Macromolecules can be taken up into vesicles via clathrin-dependent endocytosis, caveolin-dependent endocytosis, clathrin/caveolin-independent endocytosis or macropinocytosis. All of these pathways end up at the early endosome, which is subsequently routed to either the lysosome or the abluminal membrane. RMT is only complete when macromolecules are transported from luminal to abluminal membrane. Adapted with permission.^[190] Copyright 2017, Wiley.

Figure 20. Illustration of cell-mediated NP delivery to the brain. “Nanozymes” are nanomaterials with enzyme-like characteristics. Reproduced with permission.^[9] Copyright 2014, Elsevier.

Figure 21. Schematic illustration of the preparation process of erythrocyte membrane-coated polymer NPs. Reproduced with permission.^[205] Copyright 2011, National Academy of Sciences.

Figure 22. Schematic illustration of ultrasound-mediated opening of tight junctions. By introducing preformed microbubbles in the presence of a low-power ultrasound field, transient opening of the BBB can occur, followed by direct passage of drugs into the brain parenchyma. Reproduced with permission.^[216] Copyright 2014, Elsevier.

Figure 23. Schematic illustration of ultrasound-induced microbubble excitation. Stable cavitation occurs at low acoustic pressures, resulting in safe, transient opening of the BBB. Inertial cavitation occurs at high acoustic pressures, resulting in violent microbubble destruction and damage to the local microenvironment. Reproduced under the terms of the CC-BY 3.0 license.^[217] Copyright 2014, Ivyspring International Publisher.

Figure 24. Schematic representation of the transmembrane structure of BBB efflux transporters. By developing an understanding of the structure and topology of these transporters, domain-specific efflux inhibitors can be engineered and co-administered with drugs that would otherwise serve as efflux substrates. Adapted with permission.^[230] Copyright 2005, Springer Nature.

Figure 25. (A) Structure of a poloxamer (block copolymer) molecule arranged in its tri-block formation (EO-PO-EO). (B) Representation of a poloxamer micelle with a solubilized drug in its core. Reproduced with permission.^[242] Copyright 2008, Elsevier.

Figure 26. Simplified illustration of the prodrug strategy. (A) When attached to its promoiety, the drug is pharmacologically inactive until it crosses the BBB. Once it reaches the brain, an enzymatic or chemical reaction cleaves the prodrug and renders it pharmacologically active. (B) Common functional groups on parent drugs that can be modified to form prodrugs. Different promoieties are shown in green. Adapted with permission.^[273] Copyright 2008, Springer Nature.

Figure 27. Illustration of three methods for drug loading within LbL capsules. a) Drug cargo integrated into capsule wall. b) Capsule template is preloaded with cargo prior to LbL

assembly. c) Capsule is post-loaded by altering its permeability and entrapping cargo inside. Reproduced with permission.^[293] Copyright 2010, Wiley-VCH.

Figure 28. Schematic illustration of various (A) lipid-based NPs, (B) polymeric NPs, and (C) inorganic NPs. Liposome and SLN adapted with permission.^[307] Copyright 2005, Springer Nature. Micelle adapted with permission.^[308] Copyright 2004, Springer Nature. Dendrimer adapted under the terms of the CC-BY 3.0 license.^[309] Copyright 2017, InTechOpen. Polymeric nanocapsule and nanosphere adapted with permission.^[310] Copyright 2014, National Institutes of Health/Department of Health and Human Services.

Figure 29. Schematic illustration of liposome structure. Liposomes can be categorized as small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), or multilamellar vesicles (MLVs), depending upon their size and number of lipid bilayers. Adapted under the terms of the CC-BY 4.0 license.^[314] Copyright 2014, Frontiers Media SA.

Figure 30. Schematic illustration of a chitosan-coated liposome with hydrophobic drug molecules entrapped within the lipid bilayer. Adapted with permission.^[349] Copyright 2016, Royal Society of Chemistry.

Figure 31. Schematic illustration of the oscillation of free electrons in a gold nanosphere. It is this oscillation in the presence of light that generates the localized surface plasmon resonance (LSPR). Reproduced with permission.^[386] Copyright 2015, American Chemical Society.

Figure 32. Schematic illustration of various polymer-iron oxide stabilization methods.

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Figure 33. Schematic illustration of different MNP arrangements that can be used to achieve targeted drug delivery. Reproduced with permission.^[405] Copyright 2015, Elsevier.

Figure 34. Schematic illustration of the NP internalization process by opsonization and phagocytosis. Reproduced with permission.^[424] Copyright 2009, Springer Nature.

Figure 35. Schematic illustration of the effect of NP functionalization with PEG. (A) Uncoated NPs circulate through the vasculature and encounter many non-specific interactions, resulting in the adsorption of opsonins and the formation of a protein corona that ultimately leads to opsonization. (B) NPs functionalized with hydrophilic surfactants such as PEG are prevented from interacting with opsonins. This gives the NPs stealth-like properties and renders them invisible to the RES. Reproduced under the terms of the CC-BY 4.0 license.^[314] Copyright 2014, Frontiers Media SA.

Figure 36. Schematic illustration of (A) different methods of PEG anchoring on NP surface, and (B) different conformations that the PEG chains can take. Adapted with permission.^[431] Copyright 2014, Elsevier.

Figure 37. Schematic illustration of (A) “naked” NP with no protective coating, (B) PEGylated NP in mushroom-like configuration, and (C) PEGylated NP in brush-like configuration. Reproduced with permission.^[431] Copyright 2014, Elsevier.

Figure 38. Schematic illustration of the structure of an amphiphilic NP self-assembled from its constituent polymers. Adapted with permission.^[499] Copyright 2016, Elsevier.

Figure 39. Schematic illustration depicting the proposed mechanism of CPP-induced NP internalization. CPPs such as TAT peptide can generate a saddle-splay curvature in the BBB's luminal membrane, enter through an induced pore, and interact with cytoplasmic actin to promote cellular uptake of the attached NP via AMT. Reproduced with permission.^[500] Copyright 2011, National Academy of Sciences.

Figure 40. Chart showing comparable brain distribution of ritonavir in mice injected intravenously with ritonavir solution, or with ritonavir-loaded NPs without any CPP attached, or with TAT-conjugated ritonavir-loaded NPs. At two weeks post-injection, TAT-conjugated NPs show an 800-fold increase in brain drug concentration compared to free drug, and a sevenfold increase compared to unconjugated NPs. Reproduced with permission.^[470] Copyright 2008, Elsevier.

Figure 41. Relative brain distribution of various formulations comprising anti-inflammatory drug Diclofenac sodium. RGD-coated magnetic liposomes showed greatest brain uptake, likely a result of immune cell-mediated localization. Reproduced with permission.^[200] Copyright 2003, Elsevier.

Figure 42. Schematic illustration of the proposed EPR effect, which is believed to facilitate the extravasation of NPs into some inflamed and tumor tissues. Reproduced with permission.^[530] Copyright 2016, Elsevier.

Figure 43. Schematic illustration of the passive targeting of NPs in human tumors. The microenvironment in inflamed and tumor tissues presents significant differences between humans and mice. Reproduced with permission.^[530] Copyright 2016, Elsevier.

Figure 44. Active targeting strategies within the CNS. In order to facilitate precise localization of drugs in target tissues, NPs can be incorporated with a magnetic core to permit magnetic guidance to target regions. They can also be functionalized with (A) pH-responsive linkers, (B) thermally-responsive linkers, (C) redox-responsive linkers and (D) disease-specific targeting ligands or antibodies. These moieties are designed to recognize diseased tissues and subsequently trigger site-specific payload release.

Figure 45. Schematic illustration of the pH-triggered internalization of doxorubicin-loaded NPs leading to intracellular drug release. Reproduced with permission.^[541] Copyright 2011, American Chemical Society.

Figure 46. Schematic illustration of the process leading to site-specific hyperthermia-triggered drug release from temperature-sensitive MagFolDox liposomes. Reproduced with permission.^[546] Copyright 2010, Elsevier.

Figure 47. Schematic illustration of doxorubicin-loaded liposomes functionalized with redox-active ferrocene-modified phospholipids. Oxidation triggers release of the drug payload at the cancer site. Adapted with permission.^[551] Copyright 2016, American Chemical Society.

Figure 48. Examples of bispecific antibodies engineered against AD targets. (A) In all of these examples, a low-affinity anti-TfR domain mediates RMT across the BBB, permitting

more effective drug release into the brain than would a high-affinity anti-TfR domain. (B–D)

The therapeutic domain of a BsAb can be engineered against any one of several disease targets, including BACE1, A β monomers or plaques, soluble Tau aggregates, and even synaptic targets. Reproduced with permission.^[569] Copyright 2011, The American Association for the Advancement of Science.

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The blood–brain barrier (BBB) remains a major challenge to the central nervous system (CNS) therapeutic development. However, a deepening understanding of the CNS anatomy and physiology, coupled with advances in the engineering of biomedical nanomaterials, are enabling new strategies for overcoming the BBB and targeting CNS disorders. This Review examines various nanomaterial-mediated therapeutic approaches, including their advantages and limitations.

Keyword: blood–brain barrier, neurological diseases, nanoparticles, nanomaterials, drug delivery

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