Chapter

Crossing Blood-Brain Barrier with Nano-drug Carriers for Treatment of Brain Tumors: Advances and Unmet Challenges

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Abstract

Blood-brain barrier (BBB), a unique membrane barrier formed by closely stitched brain capillary endothelial cells (BCEC) with tight cellular junctions, separates brain from the circulating blood to protect it from bloodborne pathogens. BBB greatly limits the entry of chemotherapeutics to brain, and in consequence, it is a major obstacle for treating brain tumor. Advances in designing efficient nano-drug carriers are opening new avenues for overcoming this uphill systemic challenge. This book chapter describes current understanding of nanocarriers-mediated noninvasive drug targeting to brain tumor. Design principles behind the construction of the most promising recently designed receptor and transporter selective nano-drug carriers for combating brain tumors have been highlighted.

Keywords: blood-brain barrier, brain tumor, nanocarrier, drug delivery, nanomedicine

1. Introduction

Gliomas are the deadliest primary central nervous system (CNS) neoplasms arising from rapid proliferation of glial cells, the non-neuronal cells present in brain. Based on histopathologic features and progression of the disease, gliomas are classified by WHO into four grades: grade I (pilocytic astrocytoma), grade II (astrocytomas and oligodendrogliomas), grade III (anaplastic astrocytomas and oligodendrogliomas), and grade IV (glioblastoma multiforme). The low-grade (I and II) glial tumors often evolved with time into high-grade (grade IV) glioblastoma multiforme (GBM). However, irrespective of their grading, glial tumors almost invariably exhibit marked infiltrative growth pattern with tumor cells traveling long distances away from their origin into the surrounding healthy brain tissue. Furthermore, they are highly proliferative along with their significant angiogenic potential and resistance to apoptosis. GBM has a median survival of 14–17 months post diagnosis and only 3–5% survivability beyond 5 years. Current standard of care to treat gliomas includes safe surgical resection of the tumor followed by radiotherapy and chemotherapy. Despite significant advances in the cancer treatment, therapeutic success against gliomas remained an unmet challenge mainly because of their diffusive infiltrating growth pattern with rapid proliferation rate

and physiological location, which made them difficult to cure completely either by surgical excision or application of radiotherapy/chemotherapy [1, 2]. More often than not rapid recurrence of tumor ensues. Poor drug accumulation in glioblastoma tissue, unfavorable pharmacokinetic behavior, and toxicity to off-target organs are retarding the clinical success of systemic chemotherapy of glioblastoma.

1.1 Physiology and anatomy of human brain

Toward developing an effective therapeutic strategy for combating glioblastoma, a basic understanding of brain physiology is very important. Brain is an integral part of the central nervous system. Primary brain cells include equal number of neuron and glial cells [3]. Where neurons connect different body parts by transmitting information, glial cells provide structural support and protection to the neurons. Both cells together organize into specialized structures, which can be classified as gray matter (dominated by cell bodies) and white matter (dominated by axons). The three major subdivisions of human brain are the cerebrum, cerebellum, and the brain stem (**Figure 1**) [4]. The largest part cerebrum is divided into the right and left hemispheres along the mid-sagittal plane. These hemispheres are made up of an outer layer of gray matter named as the cerebral cortex responsible for language and information processing. Cerebral cortex cells communicate with each other and with the spinal cord via the underlying cerebral white matter. Communication between the two cerebral hemispheres primarily occurs via a major white matter tract called the corpus callosum. The cerebellum contains a similar kind of gray and white matter organization but at a smaller scale. It functions primarily to control balance and coordinated movement. The brain stem, responsible for involuntary functions such as heart rate and breathing, connects the brain to the spinal cord. It also contains both gray and white matter regions. However, unlike in cerebrum and cerebellum, they are not organized into inner and outer layers. Most of the brain tumors occur in the parenchymal space of the cerebrum [5]. However, getting drugs into the brain is much more difficult than that into other body tissues as brain tissue is highly protected both externally and internally. Skull externally protects brain tissue and regulates intracranial tissue pressure by constraining the volume [6], which limits the regional mode of drug delivery to brain. Brain is internally protected by the blood-brain barrier (BBB), which prevents random entry of

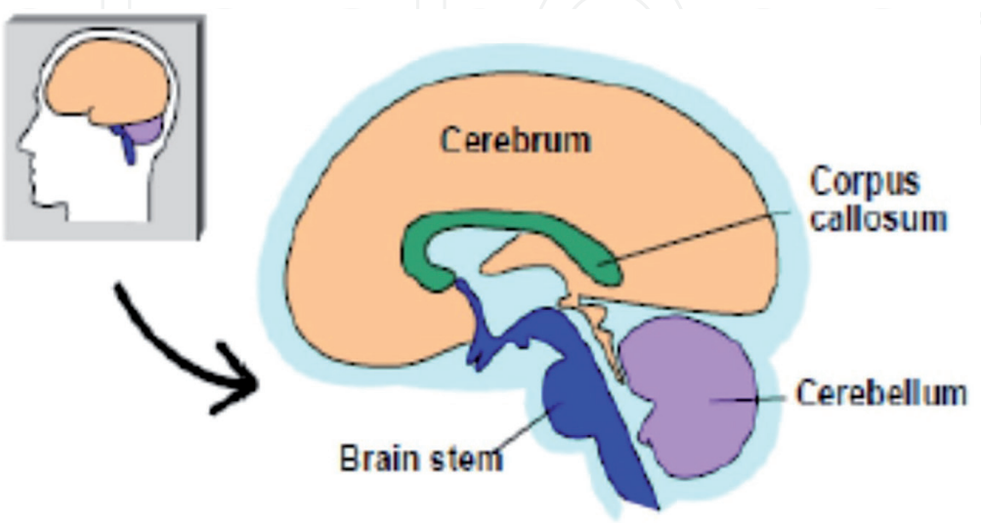

Figure 1.
Basic anatomy of human brain; cerebrum, cerebellum, and brain stem are the three major subdivisions of human brain.

molecules from blood circulation into brain tissue making delivery of systemically administered drugs to brain an arduous task.

1.2 Blood-brain barrier (BBB)

Blood-brain barrier (BBB) is a highly selective and protective membrane barrier that separates the central nervous system (CNS) and prevents entry of random substances from circulating blood to CNS. It protects brain from bloodborne pathogens and maintains the homeostatic regulation of the brain microenvironment [7, 8]. The presence of BBB was first presumed in 1885 when the German bacteriologist Paul Ehrlich found no trace of a water-soluble aniline dye in the brain and cerebrospinal fluid (CSF) after injecting it in the peripheral circulation while it was found in other organ. It was initially assumed that the dye has bonding affinity toward other organ except the brain and CNS [9]. However, in 1913 when E. Goldman, a student of Ehrlich, repeated the same experiment and performed additional experiment by injecting the dye into CSF of dogs, the presence of dye was found in the CNS including brain and the spinal cord only when it is injected in CSF of dogs [10]. Then it strengthened the hypothesis, previously suggested by Bield, Kraus, and Lewandowsky, that there must be a barrier that is preventing the transfer of dyes between blood and brain [7]. In 1937, after the invention of the scanning electron microscopy (SEM), the actual membrane barrier was observed.

The BBB is primarily composed of a continuous layer of brain capillary endothelial cells (BCEC) securely interconnected by tight junctions and adherens junctions, a basement membrane, pericytes, and perivascular astrocyte end-foot processes. The BCEC cells are highly interconnect via tight junction to form a thin wall-like structure (~200 nm), which is from the luminal side (BBB facing blood), covered by heparan sulfate proteoglycans, laminin, collagen type IV, and other extracellular matrix proteins. In comparison with the endothelial cells at the peripheral micro-vessel, BCEC differs in majorly two ways. Firstly, due to the presence of tight junction, the connection between endothelial cells at BBB is ~50–100 times tighter than endothelial cells at the peripheral micro-vessel wall, and there is no fenestration in BBB [11–13]. In addition, BBB endothelial cells have very few pinocytotic vesicles unlike endothelial cells in the rest of the body. As a consequence, transport of nutrients from the blood to the brain requires energy-dependent active transport pathway indicating the presence of ~5–6 times more mitochondria. BBB endothelial cells offer an enzymatic barrier due to the presence of proteolytic enzymes including c-glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase [14]. This enzymatic barrier has the capability to break down the neuroactive bloodborne solutes and drugs. The pericytes are covering 20% of the outer surface of endothelial cells. The primary function of pericytes is to form two basal laminas (BL1 and BL2) together with the smooth muscle. The BL1 is the distinct extracellular space between endothelial cells and pericytes, whereas BL2 is the extracellular matrix between pericytes and the glial end feet bounding the brain parenchyma. They are responsible for the regulation of the blood flow in the brain capillary through contraction and relaxation. Astrocytes are a type of glial cells in the CNS with an important role in BBB. The end feet of astrocytes form a complex supporting network surrounding the endothelial cells, which connects endothelial cells with neurons and microglia [15]. This complex network structure of astrocyte end feet is essential for proper function of BBB. Astrocytes can also enhance the level of tight junction proteins, which is crucial for the structural integrity and low permeability of BBB [16]. Moreover, it protects BBB from oxidative stress by inducing anti-oxidative activity in the endothelial cells.

Other than the role in BBB, astrocytes are also essential for maintaining brain homeostasis, injury protection, clearing of synapses. For all the versatile roles, astrocytes are considered as the primary workhorse of the CNS [17].

Other two notable cellular components of BBB are basement membranes and microglia. Basement membranes are composed of extracellular matrix proteins. It provides structural support for endothelial cells and separates themselves from the inner brain tissue [18]. Microglia are a subtype of monocyte cells present throughout the brain and spinal cord [19]. They primarily help in immune defense and maintaining CNS [20]. In addition, current evidence indicates that the activated microglia can enhance the expression of tight junctions, which helps in maintaining the integrity and proper function of BBB [21].

Other than the cellular component in BBB, there exists three types of intercellular junctions, which are responsible for the extremely tight connection between two neighboring endothelial cells: tight junction, adherens junction, and gap junction (**Figure 2**). Tight junctions are formed by many transmembrane proteins and cytoplasmic proteins. Junction adhesion molecules (JAMs), occludins, and claudins are some examples of transmembrane proteins, whereas cytoplasmic proteins include zonula occludins (ZO), cingulin, afadin, calcium/calmodulin-dependent serine protein kinase (CASK), etc. JAM proteins in BBB are expressed by endothelial

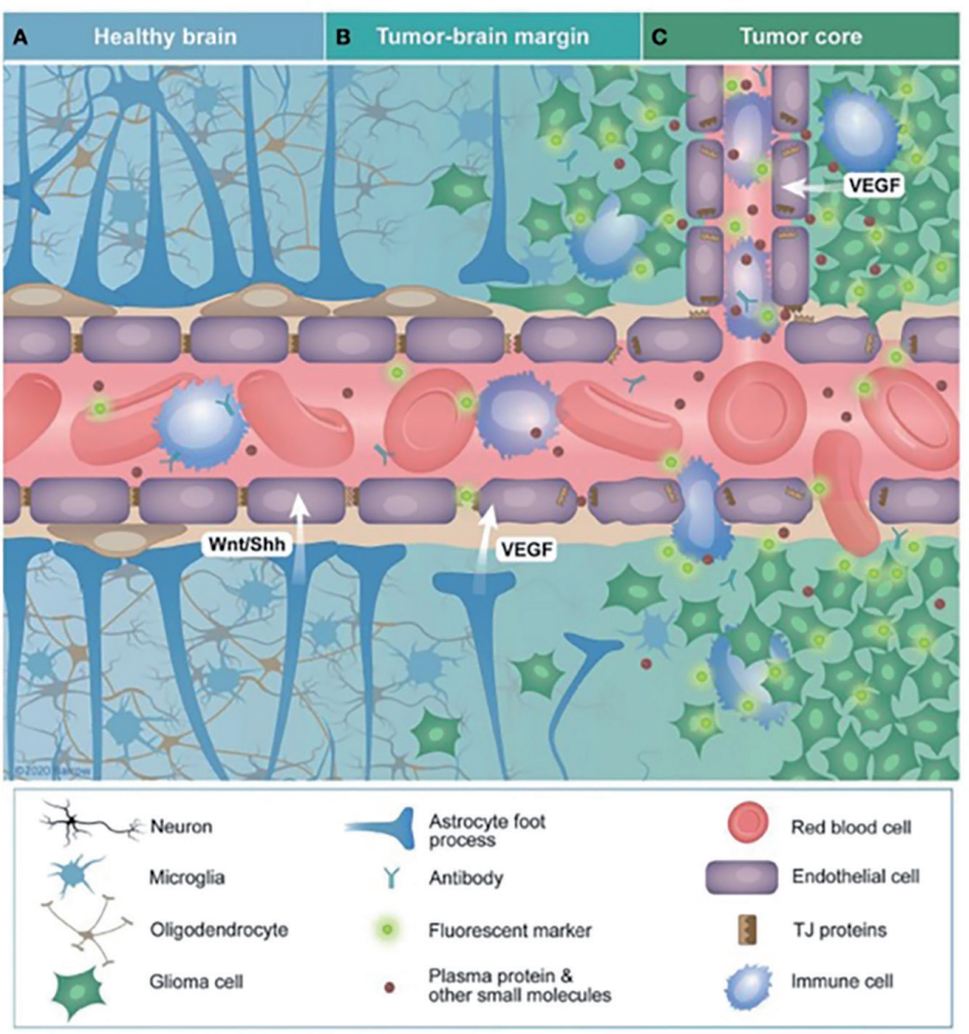

Figure 2.
Characteristics of the blood-brain (BBB) in healthy and glioma bearing brain (blood-brain tumor barriers). (A) Healthy BBB selectively permits entry of solute from blood circulation to brain parenchyma, (B) in tumor margin zone, tight junctions become weak, tumor cells penetrate or rupture BBB, (C) at tumor core, BBB structure is greatly disrupted (adopted from Ref. [22]).

cells and also expressed by leukocytes and platelets. They are highly localized on the tight junctions of BBB [23, 24] and control endothelium permeability, leukocytes migration, and cell polarity [25]. The extracellular domain of JAMs mediates the adhesive interaction between the endothelial cell and platelets as well as interaction with the leukocytes, whereas the cytoplasmic domain of JAMs interacts with various tight junction associated proteins such as ZO-1 and AF-622 [24, 25]. Claudins and occludins are the most crucial transmembrane proteins in the tight junctions of BBB [15, 26]. Claudins are small transmembrane proteins of ~27 kDa at the BBB. The extracellular domains of claudins built the tight junctions among adjacent endothelial cells and seal the paracellular cleft, whereas its intracellular parts connect to the actin filaments. Another type of transmembrane protein is occludin, which is expressed by brain microvascular endothelial cells and exclusively localized at the tight junctions. Occludins have similar function of claudins [27] (**Figure 3**). Besides these abovementioned transmembrane proteins, several other cytoplasmic proteins also contribute to constituting the intact tight junction structures.

Adherens junction is another type of junction that is crucial for the structural integrity of interendothelial cell connections and proper assembly of tight junction proteins. Any alteration of the adherens junction leads to the BBB disruption [29]. Gap junction is newly invented junction located between the tight and the adherens junction. Structurally it is an intercellular channel that connects to endothelial cells. Gap junctions allow the exchange of ions, small metabolites, and metabolic signals between adjacent endothelial cells in BBB and thereby play crucial role of maintaining tissue homeostasis in BBB [30]. In addition, gap junction also regulates permeability of BBB by interacting with scaffolding proteins ZO-1 via afadin-6 protein. Overall, the presence of tight junctions between endothelial cells significantly

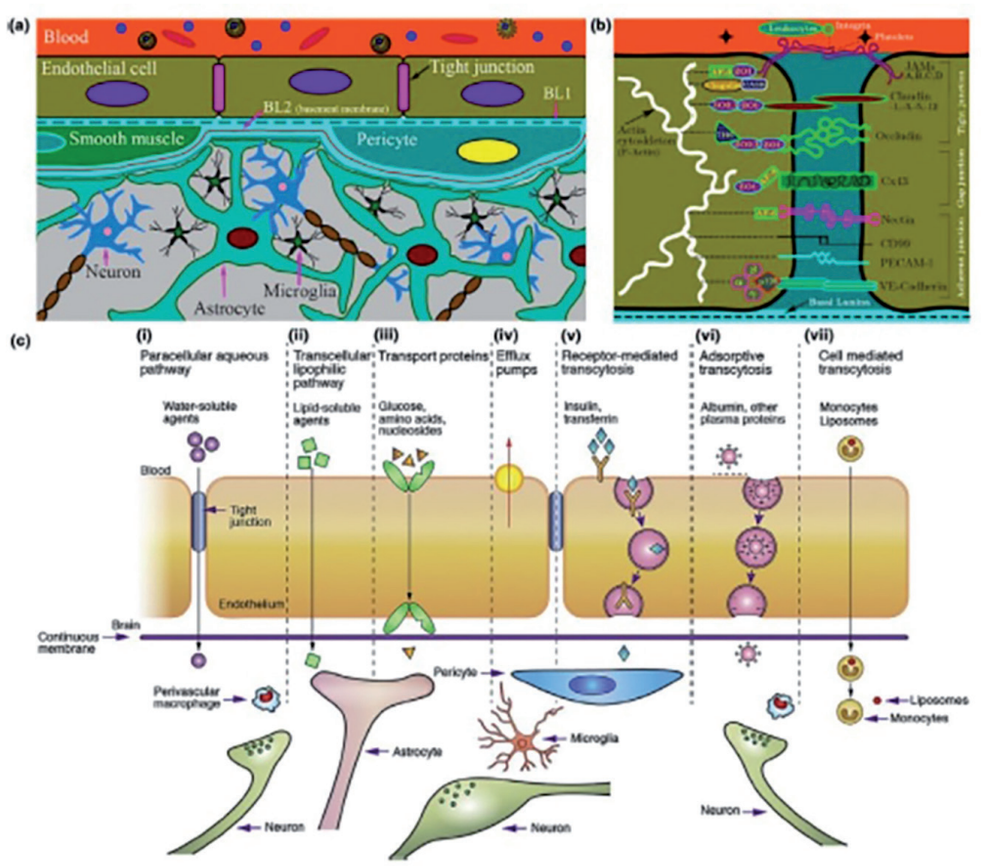

Figure 3.
(a) The cellular components of BBB; (b) structure of junctions at the BBB; (c) transport routes across the BBB (adopted from Ref. [28] with permission).

restricts the random exchange of substances through the BBB. Also, x there exists high electrical resistance (1500–2000 Ω cm^2) between the endothelial cells caused by the encapsulation of capillaries by the astrocytes and pericytes [31].

In the presence of a primary or secondary brain tumor, structural integrity of BBB is disrupted during tumor progression and then the BBB in glioma-bearing brain is named as the blood-tumor barrier (BTB) [32]. BTB is characterized by loss of junctional proteins in endothelial cells, loss of astrocytic end feet and neuronal connections, aberrant distribution of pericytes, and tumor vascularization, which greatly hampered the structural integrity of BBB with progression of glioma [33, 34]. The compromised structural integrity in BTB allows circulating immune cells, e.g., T cell and peripheral monocytes to enter in brain tumor area [35]. Notably, although BBB is disrupted at the tumor core, it may retain its characteristics intact in other area of brain and still act as barrier there. For instance, with an average-size tumor about 10% may have open junction and 30% may have fenestrations, which permits 330 kDa or smaller nanoparticles (NPs) through it [36, 37]. BTB retains the characteristics of expressing efflux transporters in endothelial cells and tumor cells and often exhibits higher expression of some receptors favoring the tumor growth such as GLUT1 and BCRP [38].

1.3 Crossing the bar: transport pathways across the blood-brain barrier

Current approaches of drug delivery to brain include regional and systemic mode of delivery. In regional mode of delivery, therapeutics are directly injected (intracranial injection) into the brain by stereotactic surgery. However, this method results only in localized delivery of drug around the injection site with limited penetration into the brain parenchymal space. Moreover, stereotactic surgery of brain involves drilling of the skull, which is too invasive for human therapy. Systemic mode of delivery via intravenous administration is the ideal noninvasive therapeutic modality to deliver chemotherapeutics into the brain. Rich vascularity of the brain, with blood capillaries spreading virtually throughout all the brain cells, enables efficient assimilation of chemotherapeutic agents into the brain parenchyma provided the therapeutics could cross the BBB [39]. A great deal of effort, therefore, is presently focused on development of BBB-permeable therapeutics. Recent trends to overcome BBB are directed toward exploitation of some active transporter expressed in BBB for supplying nutrients to maintain brain homeostasis. The transports of molecules across BBB can be broadly classified into two ways, passive transport and active transport (**Figure 4**). The passive transport is nonspecific and energy (ATP)-independent process, for example, diffusional transport via paracellular or transcellular transcytosis and passive accumulation of drugs in tumor vasculature via enhanced permeability and retention effect (EPR). On the other hand, the active transport routes such as receptor-mediated transcytosis (RMT), carrier-mediated transcytosis, adsorption-mediated transcytosis (AMT), and cell-mediated transcytosis, all of which require adenosine triphosphate (ATP).

In paracellular diffusion, solute molecules enter the brain through the space between two adjacent endothelial cells. Only aqua-soluble small molecule with molecular weight less than 500 Da can pass through the paracellular space driven by the negative concentration gradient from blood to brain [41]. Modulation in the tight junction can enhance paracellular diffusion rate although it may expose brain parenchyma to unwanted substances [42]. In transcellular diffusion, the solute particles diffuse through the endothelial cells from blood to brain driven by negative concentration gradient similarly. However, for transcellular diffusion, the solutes should be non-ionized, with desirable hydrophilicity and lipid solubility. For instance, steroid and hormones cross BBB via transcellular diffusion.

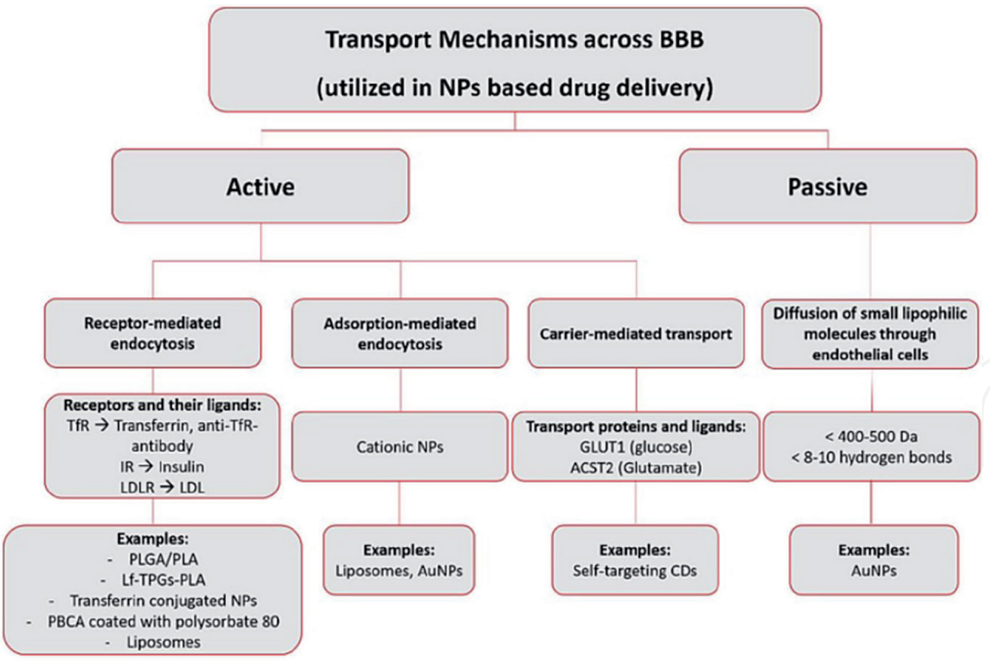

Figure 4.
Different types of transport pathway across BBB (adopted from Ref. [40] with permission).

Among active transport systems, receptor-mediated transcytosis (RMT), where the particles cross BBB by using the receptors expressed on apical surface of the BBB endothelial cells, is an important pathway for transporting drugs to the brain. Currently, it is widely being used for nanocarrier-mediated targeting of drug to the brain. The mechanism of RMT relies on endocytosis where the ligand specifically binds to the receptor followed by formation of an intracellular vesicle via membrane invagination. The membrane invagination occurs either via clathrin or via caveolae-mediated mechanism. In both cases, after vesicle formation, the vesicles are detached from the membrane and trafficked to three different fates. The major portion is directed to the basolateral membrane, fuses, and releases their payload, whereas some vesicles are recycled to the apical side, and some other undergoes lysosomal degradation. Transferrin receptors, low-density lipoprotein receptors, lactoferrin receptors, etc., are some of the most commonly targeted receptors for drug delivery to brain tumor.

Carrier-mediated transcytosis or transporter-mediated transcytosis is another active transport mechanism across BBB. Nutrients such as glucose, amino acids, etc., are transported via specific transporter protein. In this process, nutrient molecule first binds to the specific transporter at the blood side and then the transporter protein undergoes some conformational changes to transfer the nutrients molecules into the brain side. Large amino-acid transporter (LAT) and glucose transporter isoform (GLUT-1) are examples of such transporter. High specificity of the ligand-transporter interaction of this process limits its applicability in transporting large-molecular drugs.

Charged nanoparticles or macromolecules generally cross BBB via adsorption-mediated transcytosis (AMT), which uses the electrostatic interaction between the positively charged nanocarriers and the negatively charged cell surface of endothelial cells facing blood side. In this process, the interactions are nonspecific, and many nanoparticles can be delivered. However, this nonspecific method of nanoparticles transport may also lead to accumulation in other organs under systemic settings.

Besides the transport routes mentioned above, cell-mediated transcytosis can also be used for drug delivery across BBB. This approach depends on exploiting

the immune cells including neutrophils, macrophages, and monocytes, which are capable of crossing BBB in both healthy and diseased brain. In this strategy, drugs are first loaded into liposome followed by internalization of such liposome in immune cells circulating in the blood. Then those immune cells cross the BBB and migrate to the inflammation sites in the brain by diapedesis and chemotaxis. Cell-mediated transcytosis is also named as 'Trojan horse" strategy.

Despite the presence of transporters or receptors mentioned above, drug delivery across BBB is still challenging due to the presence of tight junction as discussed earlier. This challenge of drug delivery across BBB is further enhanced by some efflux pumps present at the luminal side of brain capillary endothelial cells. The efflux pumps are protein complex in the endothelial cell surface that expel out the hydrophilic anticancer drug molecules such as doxorubicin, daunorubicin etc., against the negative concentration gradient (from blood to brain) in ATP-dependent pathway. Those pumps also prevent accumulation of hydrophobic drugs in the brain capillary endothelial cells by mitigating cellular uptake. P-glycoprotein (P-gp) is class of multidrug resistance proteins, which acts as an efflux pump in drug-resistant tumor. Therefore, regulation of efflux pump at BBB is also another potential strategy for delivering drug to brain tumor, although the efflux pumps positively impact healthy brain by protecting it from harmful neurotoxin.

1.4 Nanoparticles for drug delivery across BBB for combating glioma

Various approaches have been developed to enhance accumulation of chemotherapeutics across BBB. They include both invasive methods such as post-surgical local delivery into the brain [43, 44], convection enhanced delivery [45, 46], and noninvasive method, e.g., temporary opening of the tight junctions by external energy [47], and nanoparticles-mediated delivery [48]. However, application of external energy such as ultrasound [49], osmotic pressure [50], or microbubbles [51] to open BBB via temporary disruption of tight junctions is risky. They hamper integrity of the BBB making CNS susceptible to unwanted toxins or an uncontrolled influx of medicines [52]. To this end, nanoparticles (NPs) drug delivery through the BBB, although challenging, holds significant promise to achieve a reasonable concentration of chemotherapeutics in brain tumor and to avoid unwanted off-target toxicity in other organs.

Over the past few decades, many types of nanocarriers including polymeric, inorganic, liposomes, etc., have been explored for delivery of chemotherapeutics such as small molecules, nucleotides, peptides, proteins to brain tumor. Such NPs are designed to load drugs efficiently, to selectively deliver the payloads to brain tumor crossing the BBB or BBTB by avoiding opsonization followed by clearance by the reticuloendothelial system (RES). Delivery of the NPs across the BBB is broadly mediated by two ways: passive accumulation of plain nanocarriers and active targeting of the BBB or BBTBs via nanocarrier decorated with targeting ligand on their exo-surface [53].

Among polymeric NPs, poly(butylcyanoacrylate) (PBCA) NPs are the first (in 1995) to be used for drug delivery across the BBB [54]. Surface modification of the PBCA NPs by coating with a surfactant polysorbate 80 was reported to enhance their cellular uptake in human and bovine endothelial cells by 20-fold compared with the conventional NPS [55]. The surface coating of PBCA NPs with polysorbate 80 causes absorption of plasma apolipoprotein E (Apo-E), which further enables recognition of the coated NPs by low-density lipoprotein (LDL) receptor expressed in the brain endothelial cells. Thus, the polysorbate 80 coated PBCA NPs are internalized by the brain endothelial cells through LDL receptor-mediated endocytosis [56]. Since then, polysorbate 80 coated nanoparticles of PBCA or other polymers such as PLA, solid lipid nanoparticles (SLNs), SPION NPs are being used to deliver

Nanoparticles	Chemotherapeutics	In vivo model	Outcome	References
Polysorbate 80 coated PBCA nanoparticles	TMZ	Biodistribution in healthy rats	Enhanced uptake of TMZ in brain	[57]
	DOX	Biodistribution in glioma models	Enhanced DOX accumulation in tumor tissue	[58]
	Gemcitabine	Survival analysis in rat glioma model	Prolonged survival of glioma bearing rat	[59]
PLA NPs coated with polysorbate 80	TMZ	Pharmacokinetic and biodistribution in rats	Enhancement in half-life of TMZ with higher deposition in the brain	[60]
SLN NPs coated with polysorbate 80	CPT	Pharmacokinetic and biodistribution in rats	Increased brain accumulation of CPT	[61]
SPION NPs coated with polysorbate 80	DOX	Biodistribution and efficacy in C6/ Sprague Dawley glioma model	Enhanced brain accumulation of SPION and increased anti-tumor efficacy under magnetic field	[62]

Table 1.
Nanocarrier-mediated passive targeting of drug to brain tumor.

different drugs including temozolomide, doxorubicin, gemcitabine, etc., to brain tumor that are listed in **Table 1.**

Solid lipid nanoparticles (SLNs) have also been explored for drug delivery across BBB into glioma via passive lipophilic interaction. Such NPs are demonstrated to stabilize therapeutics such as temozolomide, or RNA-based therapeutics from non-enzymatic degradation in the blood stream. Such NPs also prevent rapid clearance from blood circulation and enhance therapeutics efficacy [63–65]. Also, the positively charged nanoparticles enhance cellular uptake in BBTB cells or glioma cells [65]. In addition, such SLNs and liposomes increase the circulation time and mediate better accumulation in brain tumor via EPR effect [66]. It is worth mentioning here that such NPs are often nontoxic, and they do not hamper BBB integrity, which is consistent with the observed insignificant changes in expression levels of BBB junction proteins occludin and claudin-1 (analyzed by Western blot) in the BBB cells following SLN administration [67]. Similarly, SLNs coated with surfactants such as polysorbate 80 or Brij 78 also enhance BBB permeability and improve drug accumulation in glioma-bearing rat brain [67, 68].

Cell penetrating peptides (CPPs) are also explored for facilitating drug delivery across BBB. For instance, pegylated liposomes decorated with CPP CB5005 in their exo-surface showed better penetration to glioma cells, delivered DOX, and enhanced the survival of animals xenografted with glioblastoma [69]. Other than polymeric NPs and SLNs, additional nanoparticles that have been used for passive delivery of drugs across BBB are listed in **Table 1**.

The most widely used approaches for drug delivery across the BBB is **active targeting** of some receptor or transporter expressed in BBB or BBTB by nanoparticles exo-surface of which is decorated with targeting ligands of such receptors/transporters (for achieving RMT or AMT to cross the BBB).

Several receptors including transferrin, LDL, GLUT1, integrin receptors, nicotinic acetylcholine, etc., have been employed during the past decades. Transferrin receptor (TfR) and LDLR are the most widely used receptors to facilitate BBB crossing of NPs due to the high affinity of their ligands transferrin and LDL. For instances, using an *in vitro* BBB model, Chang et al. have demonstrated that the uptake of TfR-coated PLGA NPs is 20 times higher than that of non-coated PLGA NPs, and the uptake is mediated via receptor-mediated endocytosis (RMT) [70]. Many other NPs such as gold nanopartcile (AuNP) [71], SPION [72], etc., have also been explored for drug delivery to glioma. For example, conjugation of carbon dots (CDs) with transferrin increased the efficiency of DOX delivery into brain tumor [73]. Transferrin receptor-mediated drug delivery across BBB is reviewed in detail elsewhere [74]. However, the major limitation of using transferrin as a ligand for TfR is that the endogenous transferrin competes with transferrin-tagged NPs for the receptor binding leading to reduced cellular uptake and compromised efficacy of the NPs. To overcome it, antibodies against TfR (such as OX26, R17-217 and 8D3, etc.) that bind TfR at different location other than transferrin are now being used as ligands to graft exo-surface of the NPs. These antibodies exhibit different level affinity and different organ selectivity for the same receptor. For example, uptake of 8D3 in brain is higher than that of R17-217 while both exhibit selectivity toward TfR expressed on the brain over that on the kidney [75].

1.4.1 Transferrin receptor

Transferrin receptors (TfRs) are attractive target for nanocarrier-mediated drug delivery to the brain. TfRs are of two subtypes, TfR1 and TfR2, with high homology in their extracellular domain. TfRs are associated with controlling the extracellular iron levels by using their natural ligand transferrin, which bind to iron directly. These receptors are highly expressed in luminal membrane of brain endothelium and overexpressed in glioma tissues, which make them attractive target for NPs-mediated glioma therapy. There are many reports demonstrating active targeting of nanocarriers decorated with TfR targeting ligand (such as transferrin (Tf) itself, antibodies, or peptides) for combating glioma. For example, Cui et al. have developed a transferrin-conjugated magnetic silica PLGA nanoparticles (MNP-MSN-PLGA-Tf NPs) and have demonstrated that such NPs when loaded with DOX and PTX can effectively inhibit tumor growth in a intracranial U-87 BALB/c nude mice model [76]. In other studies, Tf-conjugated PEG-PLA polymeric NPs are reported to deliver TMZ, which results in prolonged survival of glioma-bearing C6 rat [77]. Guo et al. have reported enhanced glioma growth inhibition in C6 rat when resveratrol was conjugated with Tf-modified PEG-PLA NPs compared with free resveratrol [78].

Other than polymeric nanoparticles, gold nanopartciles (AuNPs), liposomes, polymersomes have also been used for TfR-mediated drug targeting to brain tumor. For instance, Dixit et al. have reported Tf-conjugated AuNPs to deliver a photodynamic prodrug, Pc 4, to mouse brain, which exhibits a significant brain accumulation after 4 h of administration [71]. To overcome the drug resistance of TMZ, Lam e al. have used combination chemotherapy of TMZ and bromodomain inhibitor. They have reported that transferrin-functionalize pegylated liposomes co-loaded with TMZ and bromodomain inhibitor decreased the tumor burden and prolonged survival of glioma-bearing mice compared with the control groups with no significant systemic drug toxicity observed [79]. Tf is also combined with other targeting ligand for double targeting to achieve a better drug targeting efficacy. For instance, a dual-targeting liposomes containing Tf and RGD (ligand for integrin receptor) at their exo-surface have been developed by Qin et al. This dual-targeting liposome

RGD/Tf-LP has shown significantly higher brain tumor accumulation compared with only Tf-conjugated liposome (Tf-LP), which exhibits further much higher brain tumor accumulation than RGD-tagged liposomes (RGD-LP) in BALB/C mice bearing C6 glioma [80]. Tf is also combined with liposome containing cell penetrating peptide TAT on their exo-surface to develop dual-targeting liposome of TAT/Tf-LP. In vivo biodistribution of coumarin-loaded liposomes reveals that the dual-targeting liposomes TAT/Tf-LP have shown significantly higher brain accumulation in comparison with only Tf-LP (1.5 times) and only TAT-LP (~2 times). The anti-GBM effect of DOX-loaded Tf/TAT-LP has been demonstrated by monitoring the survival of U87 GBM-bearing rats. Treatment of DOX-loaded Tf/TAT-LP enhances the median survival of GBM-bearing rat by 59 days where as it was only 10 days for free-DOX-treated rat [81].

Other than transferrin (80 kD protein), antibodies, antibody fragments, and peptides are also used as ligands for TfR-mediated drug targeting to brain via endocytosis and transcytosis on BBB. For instance, OX26, a monoclonal antibody (mAb) against TfR1, was used for the first time in 1992 to examine BBB-crossing ability of the antibody-drug conjugate (ABC) via TfR-mediated transcytosis. This study resulted in similar rate of brain accumulation for free OX26 and drug-conjugated OX26. Recently, Yue et al. have developed an immunomicelle where micelles are covalently linked with OX26 antibody and have demonstrated much higher BBB-crossing ability of the OX26-micelle compared with the free OX26 antibody [82]. Notably, similar two other monoclonal antibodies Ri7 and 8D3 are also developed that can target TfR expressed on BCEC although not yet used in drug delivery to the brain [83]. It is worth mentioning that antibody-toxin conjugates that target TfR have progressed till clinical trial III for anti-glioma therapy. Initially, human Tf is conjugated to a diphtheria toxin with CRM107 point mutation via thioester bond to develop Tf-CRM107 IT, which exhibits higher tumor growth inhibition in preclinical mouse model (U251 tumor-bearing mice) in a dose-dependent manner than the free toxins [84]. Later, a phase I study following intra-tumoral injection reveals no adverse effect leading to a phase II study in recurrent high-grade brain tumor patient where 35% of the patients exhibit tumor response and improved survival. Unfortunately, Tf-CRM107 fails to exhibit superior activity over the standard of care in an early phase III clinical trial, and CNS toxicity is observed, which lead to termination of this trial [85].

Despite the high targeting ability of mAb, difficulty in their preparation and purification in rigorous laboratory condition introduces hurdle in quality control of the mAbs, which further limit real application of mAb-tagged nanoparticles in drug delivery. Alternatively, short peptide fragment of the antibody with similar affinity toward the receptor has been proposed due to its small size and ease of incorporation in nano-formulation. For instance, a heptapeptide T7 (HAIYPRH) has been reported to specifically bind to TfR with high affinity (Kd = 10 nM) comparable to Tf [86]. Using T7 as TfR-targeting ligand, Jiang's group has co-delivered chemotherapeutic DOX and gene therapy agent pORF-hTRAIL to enhance the survivability of U87 tumor-bearing mice [87]. Similarly, Kuang et al., have used another pegylated nanoparticle decorated with T7 to achieve RNAi mediated in BABLB/c mice bearing U87 glioma [88]. In another interesting study by Kawamoto et al., a hybrid peptide containing a targeting peptide (T7 as TfR-targeting peptide) followed by a lytic peptide (therapeutic part) has been developed. Using this hybrid peptide in nano-formulation, this group has reported significant *in vivo* anti-tumor effect in Gl261 glioblastoma-bearing C57 mice without significant cytotoxicity [89]. All these preclinical studies indicate that targeting TfR for drug delivery across BBB may have future clinical potential.

1.4.2 Apolipoprotein receptors

Apolipoprotein receptors, specifically low-density lipoprotein receptor (LDL-R) and LDL-R-related proteins (LRP), which help lipids transportation into CNS [90] are also widely being explored to facilitate drug delivery to the brain tumor. These receptors are overexpressed on the BBB endothelium as well as glioma cells [91] compared with that in healthy brain tissue and thereby explored as potential molecular target for selective drug delivery to combat glioma. Apolipoprotein E (APOE) is the most studied ligand of such receptors used for delivering NPs to the brain. Mainly two strategies of LDL-R-mediated transcytosis are used in APOE-facilitated transport of NPs, which rely on (i) the high avidity of APOE to the NPs and (ii) the conjugation of NPs with APOE or its derivatives on their exo-surface. In the first strategy, NPs are coated with certain surfactant, i.e., polysorbate 80 (PS80) and poloxamer 188, which recruit APOE in the bloodstream for high-affinity-based association with the NPs facilitating their recognition and subsequent transcytosis by LDL-R in the brain parenchyma. Notably, J. Kreuter and his coworkers have significant contribution to reveal the mechanism of such enhanced accumulation of PS80-coated NPs. In a preliminary study, they have found that the concentration of DOX in rat brain 2–4 h post i.v. administration with DOX-loaded PBCA NPs coated with PS80 is 6 μg/g, which is much higher than that treated with non-coated NPs (non-detectable) [92]. Subsequent *in vitro* study shows that PS80-coated PBCA NPs, when incubated in plasma, adsorb apolipoproteins [93]. They further evaluated anti-glioma efficacy of the DOX-loaded PBCA NPs coated with PS80 in glioma model of Wistar rat and observed enhanced survivability of the animals without any neurotoxicity, which is associated with free DOX treatment [94]. Later on, many other groups have used such PS80-coated PBCA NPs for delivering different anticancer drugs such as DOX [94, 95], temozolomide [96], gemcitabine [97], etc., to glioma tissue. However, concerns are raised regarding use of surfactant PS80 due to the possible disruption of the BBB via modulation of the capillary's tight junctions and emerging adverse immune response [98] in rat, which lead to necessity of modification of the surfactant or directly conjugating LDL-R ligand to the NPs.

In the second strategy, the ligand of LDL-R is directly conjugated with the exo-surface of the NPs to achieve LDL receptor-mediated transcytosis. Initially, native LDL lipoprotein is presumed to be an ideal carrier due to its inherent structural core-shell features (highly hydrophobic core surrounded by a hydrophilic shell), which is capable of drug loading in addition to the targeting LDL-R binding domain. However, its challenging purification and limited drug loading capacity of the whole protein trigger development of alternate short binding sequence (synthetic peptides) as targeting ligand. For example, Grafals-Ruiz et al. reported development of gold-liposome nanoparticles grafted with ApoE peptides to achieve systemic delivery of small-nucleic acids (SNA) to glioma-bearing mouse brain [99]. In another study, Zhang et al. have conjugated peptide-22, a ligand of LDL-R, to PEG-PLA NPs for delivering paclitaxel to mouse brain, which enhances the median survivability of glioma-bearing mice [100]. Liposomes functionalized with synthetic ligand of apoB, another ligand of LDL-R, are also demonstrated to enhance drug accumulation in the brain parenchyma and to exhibit significant apoptotic effect in glioma tissue. The involvement of LDL-R is confirmed by using a LDLR inhibitor, namely suramin, which significantly diminishes the apoptotic effect of the NPs when treated in combination [101, 102].

Another receptor of the same family, namely LDL-R related proteins (LRP), has also attracted significant attention for BBB crossing not only due to its high expression level in glioma and BBB but also for its ability to act as a common receptor for other ligands such as lactoferin and melanotransferrin. Specifically, angiopep-2

peptide has attracted huge attention for its potential in glioma therapy [103, 104]. For instance, PTX-loaded PEG-PCL NPs conjugated to angiopep-2 peptide have been demonstrated to enhance survivability by 15% in U87 brain tumor mouse model [105]. Most importantly, a phase I clinical trial with angiopep-2 peptide conjugated to paclitaxel, namely ANG1005, has resulted a stable disease among eight out of 27 patients with a median of 51 days, which triggered its entry in a phase II clinical trial (NCT01967810) for examining its efficacy in patient with high-grade glioma [106]. Furthermore, ANG1005 is very recently demonstrated to exhibit clinical benefit in a phase II clinical trial for treating patient with recurrent brain metastasis from breast cancer [107]. Thus, angiopep-2 is now being considered as one of the leading ligands among the brain-targeting peptide finding its application in conjugation with different NPs to deliver different chemotherapeutics to glioma.

1.4.3 Targeting of adhesion molecules

Cell adhesion molecules (CAMs), other than mediating adhesion of cells to extracellular matrix (ECM), play important roles in tumor development and progression, tumor vasculature development, cellular migration, etc. There are five main classes of CAMs: integrins, cadherins, selectins, the immunoglobulin (Ig) superfamily, and cluster of differentiation (CD) molecules. The different expression level in healthy brain and tumor-bearing brain indicated a potential role of such CAMs in brain tumor and targeting such CAMs in glioma hold promises for antitumor therapy.

1.4.3.1 Integrin-mediated targeting systems

Integrins are heterodimeric transmembrane glycoprotein (cell surface receptors) mediating adhesion of cells to the extracellular matrix or, in some cases, to adjacent cells. Intracellularly, integrins are connected via associated proteins to the actin cytoskeleton. In the human genome, 18 α and 8 β subunits are encoded from 24 different functional integrins. Integrins are directly involved in tumor progression by facilitating angiogenesis (sprouting of new blood vessels) in tumor area and by mediating invasion and migration of both tumor endothelial cells and tumor cells. Thus, such integrin receptors are overexpressed in brain tumor cells and tumor endothelial cells and are attractive target of drug delivery for cancer therapy. RGD is an exogenous peptide ligand (Arg-Gly-Asp amino acid sequence) that can specifically binds αvβ3, αvβ5, α5β1 integrin receptors, which are generally overexpressed in tumor and tumor endothelial cells including for brain tumor. Therefore, RGD ligand is widely used for integrin receptor-mediated drug targeting into brain tumor. Zhan et al. are the first to report RGD-mediated drug targeting for combating brain tumor. They have developed cyclic RGDyK-PEG-PLA micelle for delivering PTX to brain tumor and demonstrated that the PTX-loaded micelles (cRGDyK-PEG-PLA-PTX) significantly enhanced the median survival of mice bearing intracranial U87MG tumor xenografts compared with non-targeting micelle (PEG-PLA-PTX) [108]. Similarly, Jian et al. also delivered PTX to the brain of U87MG glioma-bearing Balb/c mice using a integrin-targeting poly(trimethylene carbonate)-based nanoparticles c(RGDyK)–NP, which enhances the median survival of the glioma-bearing mice by 22 days compared with mice treated with free PTX [109]. In another study, McNerny et al., have functionalized poly(amidoamine) (PAMAM) dendrons with cRGDyK at the surface for multivalent binding and with drug molecule to methotrexate to the focal point for achieving anti-glioma efficacy [110]. A multifunctional dual-targeting liposomal system (c(RGDyK)/pHA-LS) containing integrin receptor-targeting RGD moiety and dopamine receptors targeting p-hydroxybenzoic acid (pHA)

has also been reported by Belhadj et al. for delivering DOX to intracranial U87MG glioma-bearing BALB/c nude mice [111]. Peiris et al. have developed a nanochain conjugated to cyclic RGD peptide for delivering CNS-1 tumor-bearing athymic nude mice. A 2.6-fold higher DOX accumulation was observed when mice are treated with targeting NPs compared with that of the non-targeted counterpart [112]. Another chemotherapeutic agent Epirubicin is also loaded to an integrin-targeting micelle via a pH-sensitive hydrazone bond (cRGD-Epi/m) by Quader et al. High concentration of epirubicin is observed in brain tumor when mice are treated with cRGD-Epi/m, which eventually lead to inhibition of intracranial glioblastoma growth [113].

Combination of chemotherapeutics is also delivered using integrin receptor-targeting RGD ligand. For example, DOX and PTX are co-delivered in mouse brain tumor by using RGD functionalized Pluronic micelle, which resulted in significant tumor accumulation following in vivo fluorescence [114]. To this end, recently our lab has reported co-delivery of WP-1066, a small-molecule inhibitor of STAT3 and STAT3 siRNA to mouse brain tumor using α5β1 integrin receptor-targeting RGDK-liposomes [115]. Cellular uptake study in the presence and absence of integrin receptor-specific antibody shows that liposomes of RGDK enter mouse glioblastoma cells GL261 via α5β1 integrin receptor. The combination of WP-1066 and STAT3 siRNA delivered by RGDK-liposomes significantly inhibits the glioblastoma growth and prolonged the survival of C57 mice bearing Gl261 orthotopic glioblastoma. Collectively, all the aforementioned reports demonstrate potential of integrin receptor-mediated drug delivery for combating brain tumor.

1.4.3.2 Selectin-targeted nanocarriers

Selectins are single-chain transmembrane proteins including E, L, and P selectins, which are involved in cell adhesion via binding of sugar polymers. Selectin has distinct role in tumor inflammation and progression. Tumor cells exhibit cell tethering and rolling via selectin-dependent recognition of carbohydrates ligands to enhance distance during migration. An overexpression of E-selectin is observed in endothelial cells of high-grade glioma, although their definite role is not well established yet. Recently, Ferber et al. have reported that p-selectin is overexpressed not only in tumor endothelial cells but also in glioblastoma cells [116]. Using a dendritic polyglycerol sulfate (dPGS) nanocarrier, this group has delivered paclitaxel (PTX) in combination with a peptidomimetic of the anti-angiogenic protein thrombospondin-1 (TSP-1 PM) to inhibit the tumor growth *in vivo* using both murine and human orthotopic GB mouse models.

1.4.3.3 Connexin-targeted nanocarriers

Connexins, four-transmembrane glycoproteins, are major constituents of gap junction channels. Six connexin subunits assemble to form a hemi channel in the plasma membrane that docks with another such hemi channels of the adjacent cells to assemble the tight junction and mediate cell-cell interaction. A membrane protein connexin 43 (Cx43) is preferentially expressed in brain tumor and peritumoral area. To achieve Cx43-targeted drug delivery, Nukolova et al. have developed Cx43 mAb-conjugated nanogels loaded with cisplatin and using a C6 glioma model, the authors have shown that these nanogels can effectively inhibit tumor growth and significantly enhance the survival of animals while reducing the systemic toxicity of cisplatin [117]. In addition, the same group also functionalized this Cx43-targeting nanogels with another antibody of brain-specific anion transporter (BSAT1) to achieve additional tumor growth inhibition efficacy via dual targeting [118].

1.4.4 Other receptor and transporter-mediated targeting systems

Beyond these receptors mentioned above, there are many other receptors or transporters protein such as insulin receptor, acetylcholine receptor, glucose transporter (GLUT), large amino acid transporter-1, organic cation transporter OCT3 and OCTN2, etc., are expressed on BBB or on tumor cells that are explored for nanocarrier-mediated drug delivery to brain tumor. For example, Zhang et al. reported PEGylated immunoliposomes (PILs) modified with 83-14 mAb to the human insulin receptor to target gene that EGFR gene (which plays a major role in brain tumor progression) in U87 cancer cells [119], and later the same group modified the nanocarrioers with additional transferrin receptor for achieving RNAi in mice intracranially xenografted with human U87 glioma [120]. Nicotine acetylcholine receptors (nAChRs) expressed on BCEC are also targeted for delivering chemotherapeutics to brain tumor. To this end, Saha et al., from our group, have developed a nicotinylated liposomes to deliver small-molecule STAT-3 inhibitor WP-1066 for combating mouse glioblastoma [121]. The same group has also developed another BBB-crossing liposomes grafted with amphetamine at their exo-surface and using this liposome they deliver combination of paclitaxel (PTX) and PD-L1siRNA (RNAi agent for immune checkpoint inhibitor) to the glioblastoma-bearing mouse brain. This combination therapy is reported to enhance the median survival of mouse till 45 days while the untreated control mice died at 17 days [122]. Among transporters, glucose transporters (GLUTs) and large amino acid transporters (LAT-1) are widely used for nanocarrier-mediated drug delivery to brain tumor. During tumor progression, tumor cells continuously need supply of nutrients such as glucose and amino acids, which leads to overexpression of such transporter in glioma cells as well as BBB [123, 124]. Recently, Anraku et al. have developed a self-assembled supramolecular ~30 nm nanocarrier containing multiple glucose molecules via association of oppositely charged pairs of polyethylene glycol (PEG)-based block ionomers. A remarkable enhancement in brain accumulation of the micelle post ~15 min administration is observed, which is much higher than that for other nanoparticles [125]. Bhunia et al. from our group have reported a LAT-1-targeting liposomes containing L-DOPA on their exo-surface (Amphi-DOPA liposome) for delivering small-molecule STAT-3 inhibitor WP-1066 to glioblastoma-bearing mouse brain. A significant tumor growth inhibition is observed when mice are treated with WP-1066-loaded Amphi-DOPA liposomes compared with the untreated or non-targeting control-treated mice [124].

1.5 Conclusion and future perspectives

During the past decades, significant progressed has been made in developing nanocarriers for glioma therapy. Major focus in this research area has been implementation of different ligands or targeting different receptors and transporters overexpressed on BBB and brain tumor cells for delivering the payload to brain tumor tissue. However, less is known about the key critical design parameters of the nanoparticles facilitating BBB crossing. For example, it has been observed that nanocarriers with size 20–30 nm are most effective in BBB crossing while among the different shapes, nanorod is most efficient in BBB crossing followed by spherical nanocarrier. More detail information is needed in future regarding the role of surface potential, formulation or composition, drug loading method, etc., in facilitating transport across BBB. In addition, the factors influencing pharmacokinetic behaviors of the nanocarriers should be well studied and evaluated, which is very crucial for developing an effective brain-targeting drug carrier. The poor prognosis of GBM has also prompted to develop many new therapeutic strategies exploiting

inherent physical properties of the nanomaterials such as photodynamic or photothermal therapies and hyperthermia. However, biodegradability and nanotoxicity of such newly developed materials should be studied in detail. In this regard, liposomal or lipid-based nanocarriers exhibit reasonable safety profiles. Furthermore, as brain tumor cells are highly infiltrating, nanocarriers that only deliver the payload to tumor core via leaky BBB are not sufficient, rather an image-guided delivery of therapeutics has attracted significant attention in recent years indicating the need of developing theragnostic nanoparticles. Significant attention should also be paid in enhancing targeting efficiency of the nanocarrier, which is far away from satisfactory yet, either by increasing number of targeting ligand or using high-affinity ligands with optimum ratio.

In conclusion, the following aspects should be considered on designing efficient brain tumor targeting nanocarrier in future:

1. The small nanocarriers with multiple functionalities on the surface and with high fluorescence. The multiple anchoring site can facilitate conjugation with a greater number of same ligands or different ligands specific to multiple receptors and loading of more drug molecules. The fluorescence can facilitate the bioimaging. 2. Biocompatibility of the nanocarriers to eliminate scope of nanotoxicity. 3. Optimum circulation stability and biodegradability of the nanocarriers. 4. Accessibility by noninvasive advanced imaging technique such as magnetic resonance imaging and real-time in vivo microscopy to avoid unnecessary sacrifice of the animal. 5. Application of multiple approaches to develop multimodal nanocarriers for effective BBB penetration followed by chemotherapy and bioimaging.

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