Recent advancements in liposome-based strategies for effective drug delivery to the brain

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Running title: Liposomes in brain drug delivery

Abbreviations

NP, nanoparticles

polyethylene glycol, PEG

D-a-tocopherol polyethylene glycol 1000 succinate, TPGS

Cell-penetrating peptides, CPPs

transferrin receptor, TfR

5-fluorouracil, 5-FU

folic acid, FA

paclitaxel, PTX

p-Hydroxybenzoic Acid, pHA

tripeptide motif arginine-glycine-aspartic acid, RGD

cyclic RGD, c(RGDyK

Introduction

Disorders of the central nervous system (CNS) and tumors of the brain are challenging to treat, and they rank amongst the most common causes of death worldwide. [1]. In recent years, many attempts have been made to develop drugs and therapeutic agents for disorders of the brain and CNS. However, researches programmes aimed at the discovery and development of drugs for brain disorders have had very poor success compared with those in other therapeutic areas [2]. The major obstacles encountered in the development of drugs for the treatment of CNS disorders are the complexity of the brain and the impermeability of the blood-brain barrier (BBB). The BBB serves to protect the brain from damage caused by drugs and chemicals by selectively allowing small, lipid-soluble molecules to pass through the endothelial cell membrane while preventing the transfer of most drugs, peptides, large molecules, pathogens and toxins [3]. Therefore, the potential therapeutic advantages of drugs designed to act on the CNS has not yet been fully realised. Development of efficient technologies to deliver drugs across the BBB remains the major challenge to the effective treatment of CNS disorders [4]. Hence, it is necessary to develop suitable strategies to overcome these difficulties and thus to permit improved drug delivery into the brain.

Various approaches have been used to enable drugs to permeate the BBB and to reach the brain. Nanocarriers are a promising technology in this respect. Multiple types of nanocarriers with a range of sizes and physicochemical properties have been used to target therapeutic agents to the brain. These include polymeric nanoparticles, carbon nanotubes, liposomes, and inorganic nanoparticles. Of these, liposomes have been most extensively investigated as potential drug delivery agents. Liposomes are considered to be the most efficient drug delivery system in a range of diseases. Liposomes are vesicles made up of one or more spherical lipid bilayer structures. Typically, a lipophilic (hydrophobic) phospholipid bilayer surrounds an internal aqueous compartment. Liposomes are biodegradable, biocompatible, safe and nontoxic and can be used to carry both hydrophilic and hydrophobic drug molecules. They are frequently used for numerous practical applications due to their non-immunogenic nature and their ability to maintain sustained drug release in biological systems [5]. This review discusses recent development and new strategies related to liposome technologies designed to deliver therapeutic agents to the brain and, and possible applications of liposomes in the treatment of CNS disorders.

Challenges to CNS-targeted drug delivery

The BBB plays a key regulatory role in the proper functioning of the brain by acting as a permeability barrier in its blood vessels. The selective permeability of the BBB protects the brain against harmful stimuli and toxic agents and homeostasis to take place, maintaining the conditions for the complex functions of the neurons in the brain. The BBB is a complex system consisting of endothelial cells, pericytes, astrocytes, microglia and neurons [6]. The cerebral endothelial cells of the brain are considerably different from cells in other parts of the body in that they are connected by tight junctions at their margins. This prevents paracellular diffusional of aqueous agents between the adjacent endothelial cells or *via* transcellular pathway [7]. Pericytes surround the endothelium and together with the basal lamina, and astrocytic end-feet, contribute to the organization of the BBB and form the neurovascular unit (Figure 1) [6]. The endothelial cells of the BBB have an essential function in the transport of ions and other polar solutes between the blood and the extracellular fluid of the CNS [8]. Multiple mechanisms are involved in the transcellular transport pathway, ranging from simple passive diffusion to more complex receptor-mediated transport and transcytosis. Passive diffusion depends upon the physicochemical characteristics of the substance such as size, molecular weight, lipophilicity and the surface charge of the molecule [9]. A wide range of substances such as small lipophilic molecules, O_2 and CO_2 can diffuse into the BBB by passive diffusion along concentration gradients. Others materials such as nutrients, polar molecules, proteins and peptides cannot diffuse through cell membranes and therefore require specific transporters such as efflux transporters (e.g., P-glycoprotein (P-gp)), glucose transporter-1 (GLUT-1) and insulin transporter (Figure 1B) for transportation across BBB in either of directions [9]. By resulting in the efflux of different substances from the brain, these transporter proteins play a prominent role in the barrier to drug delivery. For example, P-gp results in the efflux of a large number of lipophilic drugs and cationic substances from the brain and prevents the uptake anticancer agents in brain tumors [10].

Because of the structural complexity of the BBB, it presents considerable physiological challenges to the delivery of drugs to the brain. The BBB adapts to the needs of the CNS, responds to physiological changes, and is affected by various pathological conditions. Disorders of the BBB can promote disease [9], and the BBB loses its normal function in a range of conditions including brain tumours, stroke and neurodegenerative diseases. Therefore, to treat CNS disorders, it is necessary to develop an efficient delivery system for the delivery of drugs into the brain without compromising the other functions of the BBB.

Nanotechnology for brain drug delivery

Previous efforts to treat neurodegenerative disorders through the delivery of drugs to the brain have encountered the problem of CNS drug delivery. Recent breakthroughs in BBB research provide new strategies and approaches to solving these issues. Among different approaches, nanoparticles technology have been attracted much attention and is rapidly advancing. For this purpose, a wide range of nanoparticles have been examined for their potential to enable the delivery of drugs to the brain. These include polymeric nanoparticles, liposomes, dendrimers, carbon nanotubes, gold nanoparticles and polymeric micelles [11, 12]. In general, nanoparticles (NPs) have several advantages such as the possibility of multi-functionalization, the ability to carry drug without altering their effects, specific targeting NPs to the enhancement of BBB crossing, control of drug release and modification of the pharmacokinetics of the drug. When NPs are used to deliver drug to the brain, it is the physiochemical properties of the NP which dictate the passage into the brain. The physiochemical properties of the drug enclosed in the NP are not important in this context. [11].

Moreover, NPs with a particle size < 200 nm could penetrate into tumor tissues through enhanced permeability and retention (EPR) effect. In general, NPs concentrate in tumors and have low systemic concentrations. It has recently been shown that NPs provide more opportunities for drug delivery to brain tissues by enhancing the duration over which the drug circulates in the blood [1, 11]. Here, we focus on liposomes as one of most promising NPs for brain drug targeting and delivery. In recent years, the liposomes have attracted much attention and been extensively used as important vehicles with which to transport of drugs into the brain [12, 13]. This review focuses on recent advancements in liposome – based strategies to enable drugs to cross the BBB in the management of pathologies of the brain and CNS.

Liposomes as Drug Delivery Vehicles to the Brain

Liposomes are vesicles made up of one or more spherical lipid bilayer structures. Typically, a lipophilic phospholipid bilayer surrounds an internal aqueous compartment. Liposomes are preferred over almost any other drug-carrier system due to their similar morphology to cellular membranes, and their unique ability to carry a variety of lipophilic, hydrophobic or amphipathic drugs in a single formulation. The aqueous compartment is predominantly used to encapsulate hydrophilic agents, whereas lipophilic molecules can be adsorbed on the hydrophobic bilayer. Furthermore, liposomes have several advantages including biocompatibility and biodegradability, safety and low toxicity, drug-targeted delivery and controlled drug release [14]. As mentioned above, several mechanisms are involved in transcellular pathway different material across the BBB that include passive diffusion (nonspecific endocytosis) and

active transporting (by binding to specific receptors on the surface of BBB cells). In the case of passive diffusion, the physicochemical properties of the NPs have a major role in penetration of the BBB. The size and surface properties of the liposomes play important roles in passive diffusion. Hence, alterations in the surface features such as charge and coating may influence their ability to cross biological barriers [15]. For example, it seems that cationic liposomes undergo endocytosis across the BBB cells easily owing to electrostatic interactions between positive charges on the liposomes and the negatively charged surface of BBB cells. The interaction triggers the cell internalization processes. Coating and surface functionalization of liposomes with polyethylene glycol (PEG) and other polymers may increase their circulation time in blood, preventing fast clearance through the reticuloendothelial system and improving transport of molecules to the brain. In passive diffusion, liposomes could enter the brain through passive influx and release the encapsulated drug to the target site. However, although passively targeting of liposomes is the most common method that used in clinical therapy, it suffers from several limitations, such as nonspecific uptake, low EPR effect within the brain, uncontrolled release and the crossing of BBB barriers [12]. Therefore, to enable successful delivery of liposomes to the brain, various surface modification have been made to improve and enhance their effectiveness in circumventing the barrier properties of the BBB to maintain a higher concentration of drugs inside the brain in a controlled manner [12, 13].

Surface modification of liposomes

Liposomes can transport encapsulated drug specifically (actively) or nonspecifically (passively) into cells. In the passive route, the phospholipid bilayer of the liposome exterior fuses with the phospholipid bilayer of the plasma membrane and thus the contents of the liposome enters the cytoplasm. Alternatively, the liposome can be destabilized by certain cell membrane components when adsorbed on the surface resulting in the release of the drug which then enters the cell by micropinocytosis. Liposomes can also undergo specific or nonspecific endocytosis [16]. A variety of modifications has been made to liposomes to improve the bioavailability of drugs in various regions of the brain. One of the most common strategies, is to use a variety of molecules as surface biologically active ligands (proteins, peptides and antibodies) that bind to receptors present on the surface of BBB cells and facilitate the translocation *via* receptor-mediated transcytosis or other transfection methods [12, 17]. In this section, we summarize and assess the functional roles of various modifications made to the surface of the liposomes to improve the bioavailability and concentration of drugs in the CNS.

Passive delivery of liposomes to BBB

The development of stealth liposomes, such as liposomes coated with biocompatible polymers (e.g. PEG), is an advancement in liposomal formulation which extends the duration over which liposomes circulate in the blood. This is achieved through reduced mononuclear phagocytosis and recognition by opsonins, consequently slowing down the clearance of liposomes. This helps to improve the targeting efficiency and activity of molecules that encapsulated in the liposomal formulations [18]. Vijakumar and colleagues used the passive brain targeting ability of PEGylated liposomes to enable the delivery of resveratrol to glioma tumors. Their in-vivo biodistribution study revealed that drugs loaded in PEGylated liposomes persist for longer in the circulation and accumulate more readily in the CNS than non-PEGylated liposomes [19]. In another study by the same group, D-a-tocopherol polyethylene glycol 1000 succinate (TPGS), a PEGylated vitamin E, was used to coat liposomes to increase the circulation time in the bloodstream and to enable the passive targeting of resveratrol to the brain. The TPGS coated liposomes were evaluated *in vitro* and *in vivo*. *In vitro* experiments using C6 glioma cell showed that TPGS coated liposomes have excellent cellular internalization. Additionally, a biodistribution study in rats revealed an increase in the amount of resveratrol in the brain when delivered by the liposomes as a result of passive brain targeting [20].

A study conducted by Muthu and coworkers indicated that TPGS coated liposomes loaded with docetaxel have a higher cellular uptake and cytotoxicity in C6 glioma cells compared to conventional (non-coated) and the PEG-coated liposomes [21]. Recently, verapamil and riluzole-containing PEGylated liposomes have been developed for the treatment of amyotrophic lateral sclerosis (ALS) to overcome limitations in the transport of riluzole across the BBB [22]. Using conventional formulations, the deposition of riluzole in the brain is inhibited by the efflux transporter P-gp at the BBB, this leads to treatment failure. Yang et al. developed a liposomal co-delivery system containing riluzole and verapamil (a P-gp inhibitor) for efficient transport of riluzole to brain cells. These liposomes were able to transport encapsulated drug into brain endothelial cells through endocytotic pathways. As a result, verapamil was able to suppress the P-gp efflux protein and reduce the efflux of riluzole, leading to increased concentrations of riluzole in brain cells. An in-vitro study on bEnd.3 and C8D1A astrocyte cells indicated that treatments with liposomes have a potential inhibitory effect on P-gp and decreased riluzole efflux. Hence, it seems that in this model of BBB function , the delivery of drugs to the brain can be improved by liposomes [22]. These studies revealed that liposomes coated with biocompatible polymers facilitate improved delivery of biomacromolecules to the brain, and prolong their circulation to allow passive targeting.

Active transport of liposomes to BBB

Cell-penetrating peptide modified liposomes

Active targeting or targeted delivery using specific ligands is a novel and attractive technology that can greatly improve the potential of drug delivery to the spesific site, thereby requiring a considerably reduced dose and resulting in fewer adverse effects of the drug.. To date, vrious ligands have been evaluated asnanocarriers for active targetting of the brain t. Here, we discuss some of the important targeting ligands that have been explored for for active targeting to the brain

Cell-penetrating peptides (CPPs) are short-chain amphipathic peptides which facilitate the transport of a wide variety of compounds such as peptides, proteins, oligonucleotides and drugs across cell membranes and into cells [23]. A variety of CPPs have been identified, these include natural CPPs, such as transactivator of transcription (TAT) from human immunodeficiency virus (HIV-1) and synthetic CPPs, such as mastoparan and transportan which are used extensively to deliver compounds into cells [24]. Numerous studies have shown that surface modification of liposomes with CPPs could improve the delivery of drugs to the brain. CPP facilitates the binding and internalization of CPP-liposomes to endothelial cell membranes, improves endosomal escape and increases the cellular delivery of liposomal cargo. The uptake of CPPs is mediated through endocytic pathways, but its exact mechanism is still under debate. Possible mediators include clathrin-mediated endocytosis and micropinocytosis and non-endocytic pathways, [25].

TAT (AYGRKKRRQRRR) is one of the most common cell-penetrating peptides that is used to decorate the surface of nanoparticles such as liposomes to improve the efficient intracellular delivery of liposomal cargo [26]. It has been reported that liposomes modified with TAT can deliver drugs into cells efficiently *via* a receptor-independent and transporter-independent pathway [27]. In a recent study Qin et al. used TAT to decorate the surface of doxorubicin-loaded liposome for delivery to brain glioma. The potential of TAT-modified liposomes too be used to deliver drugs to the CNS was explored using brain capillary endothelial cells (BCECs) and C6 glioma cells. The investigators demonstrated that TAT played an important role in the trans- endothelial and cellular uptake process in an *in vitro* model of the BBB, and that cellular uptake of the doxorubicin-loaded liposome was improved by TAT. An in-vivo biodistribution study in the brain revealed that the doxorubicin-TAT liposome more accumulated in the brain and the concentrations of doxorubicin in the brain of doxorubicin-TAT liposomes were found in greater abundance in the brain than unmodified liposomes. Additionally, the cardiac concentrations of doxorubicin in the group treated with the doxorubicin-TAT liposome were much lower than in the groups treated with the unmodified liposome and free doxorubicin. Thus the TAT liposome had the potential to

reduce the cardiotoxic effects of doxorubicin, The survival study on brain glioma-bearing rat demonstrated that animals treated with TAT-modified liposome survived for substantially longer than those in other groups [28].

In addition to improving the penetrative capacity on liposomes into mammalian cells, some CPPs are able to facilitate targeted delivery to specific subcellular structures such as the cytoplasm, cell nucleus, mitochondria and lysosomes [29]. Asparagines-Glycine-Arginine (NGR), a peptide that contains a vascular homing motif has been used to modify drug-loaded liposomes in order to increase their penetration into and accumulation in tumor tissues. NGR peptide was able to target CD13/aminopeptidase N, which is over-expressed on the endothelial cells of glioma, resulting in improved tumour-targeting efficiency and anti-tumor effect [30]. The peptide iNGR (CRNGRGPDC) containings three motifs including a tumor vascular antigen CD13 targeting motif, a protease recognition site and tissue penetration motif. Hence, iNGR was able to specifically recognize tumor vascular antigen CD13, penetrate into tumor vessels and reach deep tumor parenchyma through specific interaction with the receptor NRP-1 which is overexpressed on the tumor vessels and glioblastoma cells [31]. Recently, liposomes conjugated with iNGR peptide have been developed for the purpose of targeting the tumor vasculature and to penetrate across tumor blood vessels in the treatment of glioblastoma. In one study, it was demonstrated that iNGR-modified liposomes resulted in a remarkable enhancement of the cellular uptake of drug by U87MG cells and HUVECs compared to unmodified liposomes. Also, *in vivo* imaging in mice bearing glioblastoma demonstrated that these liposomes effectively accumulated at the site of the tumor and could penetrate into tumor blood vessels and tissues. Moreover, iNGR-modified doxorubicin liposomes have a greater cytotoxic effect on the tumor - more than that of unmodified liposomes and the survival time was significantly increased in an animal model of glioblastoma. Therefore, it is apparent that the modification of liposomes with the iNGR peptide enhances the penetration of liposomes into tumors and is therefore a potentially interesting means to improve anticancer therapies [32].

Receptor-mediated transportation

Transferrin receptor-mediated transcytosis

Many receptors are overexpressed on the BBB. These include receptors for transferrin (Tf), insulin and low-density lipoprotein protein. When these receptors interact with their specific target ligands, the receptor-ligand interaction promotes transport of the ligand into the cell. Thus, the surface of liposomes

could be functionalized with receptor ligands, to mediate their cellular internalization *via* BBB [33]. In recent years, liposome functionalized with ligands have been successfully used in the delivery of drug-loaded liposomes to the brain. The transferrin receptor (TfR) is a transmembrane glycoprotein that is highly expressed on the surface of brain endothelial cells and cancer cells and is involved in the transportation of iron to the brain, by receptor-mediated endocytosis [34]. TfR is the most commonly evaluated receptor in BBB targeted delivery. Transferrin is an iron-binding serum glycoprotein and it is the most specific protein that is widely used as a TfR ligand. It improves the targeting of therapeutic cargo across the BBB and increases the accumulation of drug in the brain [35]. Tf is an 80 kDa glycoprotein, it has an isoelectric point of 5.5, hence it exhibits negative charge in a solution with a pH of 7.4. It confers a negative charge on liposomes modified with Tf as compared with that of unmodified liposomes. Transferrin modified liposomes have been studied for their potential to deliver therapeutic agents to the brain. It has been demonstrated that these types of functionalized liposome (Tf-modified liposomes) have a higher affinity for brain capillary endothelial cells and significantly enhanced liposomal cargo delivery to the brain than unmodified liposomes [36, 37]. Recently, liposomal resveratrol (a natural polyphenol with anti-cancer effects), was modified with transferrin (TF) to produce Tf- resveratrol- liposomes for the purpose of drug delivery to the brain. The Tf-modified liposomes showed a significantly greater accumulation in cancer cells compared to normal human astrocytes, possibly due to overexpression of TfRs in cancer cells. Tf- resveratrol- liposomes induced significantly greater apoptosis and cell cycle arrest, in U-87 glioblastoma cells compared to free drug and drug-loaded liposome. Furthermore, in an *in vivo* study, it was demonstrated that mice treated with Tf- resveratrol-liposome had smaller tumors and prolonged survival compared to free drug and non-targeted liposome. Biodistribution studies indicated that PEGylated resveratrol-liposome and Tf- resveratrol-liposome accumulate to a greater extent in the tumors, hence, it appears that passive targeting, the EPR effect and receptor mediated transcytosis may be involved in mediating the accumulation of resveratrol-liposomes at the tumor site [38].

Moreover, Lopalco et al. indicated that transferrin-functionalized dopamine-loaded liposomes could be successfully transferred across an in-vitro model of the BBB [39]. Song and colleagues developed TF-modified liposomes to transport vincristine and tetrandrine across the BBB. They demonstrated that TF-modified encapsulated drug liposomes increased cellular uptake of drug across the BBB and induced a greater cytotoxic effects on C6 cells. Furthermore, TF-modified vincristine and tetrandrine liposomes, vasculogenic mimicry (VM) channels were significantly inhibited, cancer cell invasion was suppressed and the expression of apoptotic proteins were significantly increased. In glioma-bearing mice, treatment

with TF-modified vincristine and tetrandrine liposomes was associated with longer median survival time than the other groups [40]. In summary, all studies clearly demonstrated that transferrin is very useful ligand that can be used for the transport of NPs across BBB by receptor-mediated transcytosis

Multi-ligand functionalized liposomes

The use of multiple ligands and surface-active agents is another promising approach to enhance the efficacy of drug targeting nanocarriers. This approach could overcome several drawbacks such as receptor saturation and lysosomal degradation during endocytic uptake and thereby provide a feasible approach to yield enhanced therapeutic results. Using this approach, liposomes have been modified with more than one active ligand capable of binding to specific receptors in the BBB in order to enhance the efficiency of drug delivery [41].

CPP-TF dual functionalized liposome

An example of a dual functionalized liposome is the use of both CPPs and transferrin on the surface of the liposome. Binding and translocation of CPP-coated liposomes occurs as a result of the positive charge of CPPs and the interaction of CPP-coated liposomes with negatively charged endothelial cell membranes. The presence of transferrin on the liposomes facilitates transport *via* receptor-mediated translocation and improved penetrative effect of CPPs [42]. Lakkadwala et al. developed dual functionalized liposomes to enhance the delivery of chemotherapeutic agents across the BBB for the treatment of glioma. They modified the surface of liposomes with transferrin to target receptors, and the cell penetrating peptide PFVYLI (PFV) to enhance cell penetration [43]. In another study this group modified the surface of liposomes with transferrin and two CPPs (TAT and QLVPM) to enhance cell penetration [44]. They used the modified liposomes to promote the translocation of doxorubicin (Dox) and erlotinib (an epidermal growth factor receptor inhibitor) across the BBB in an *in vitro* glioblastoma tumor model. Tf- CPPs modified liposomes demonstrated relatively high cellular uptake and high concentrations of Dox and erlotinib in glioblastoma tumor cells. Additionally, Tf- CPPs modified liposomes enhanced tumor cell death and antitumor efficacyin an in-vitro brain tumor model [43, 44]. Recently, bifunctional liposomes containing Tf mediated receptor targeting and poly-L-arginine (PR) as a CPP were produced with the intention of delivering genes to brain. The bi-functional liposomes were more readily taken up by brain endothelial cells and had a higher transfection efficacy in primary culture of glial than the Tf liposomes. Additionally, bi-functional liposomes exhibited considerably enhanced

cell penetration in an in-vitro BBB model [42]. Using both *in vitro* and *in vivo* methods, Sharma et al. investigated multi-functionalized liposome modified with CPPs-TAT, Penetratin and Mastoparan on the transport of doxorubicin encapsulating transferrin liposomes into brain endothelial cells. This study demonstrated that the dual functionalized (CPP-Tf) liposomes were more efficiently transported across cell membranes as compared to single ligands (including Tf or CPP-liposomes). Tf-TAT, Tf-Penetratin liposomes demonstrated efficient delivery of doxorubicin across the brain endothelial barrier in an in-vitro model of brain tumor. Tf-Penetratin liposomes demonstrated greater cellular uptake and transport of doxorubicin *in vivo* and *in vitro* in comparison to Tf-TAT liposomes due to higher cationic charge of penetratin. Mastoparan peptides improved cellular uptake of Tf-liposomes *in vitro* and have a minimum endothelial transcytosis owing to lower cationic charge. It was also demonstrated that Tf-Mastoparan liposomes have a higher cytotoxicity and hemolytic activity and faster clearance, therefore leading to lower transport of doxorubicin *in vivo* and *in vitro* in comparison to other Tf CPP liposomes. Tf-mastoparan liposomes have a greater uptake by liver, spleen and lungs and therefore, have an easier availability for transport to brain [45].

Recently, the effect of dual-functionalized liposomes conjugated with the CPP peptide, penetratin and TF was investigated to enhance the transport of 5-fluorouracil (5-FU), across the BBB into tumor cells. It was reported that the co-modification of liposomes with Tf and penetratin improved the cellular uptake of the liposomes in U87 glioblastoma cells and a monolayer of bEnd.3 cells. The investigators suggested that the cationic charge of penetratin could reduce the negative charged on Tf and thereby facilitate the binding and internalization of liposomes. In addition, 5-FU-loaded dual-functionalized liposomes was able to induce significantly higher apoptosis in U87 cells and were associated with enhanced transport across the brain endothelial barrier. Aditionally, Tf-penetratin modified liposomes loaded with 5-FU were able to undergo endocytosis, thereby delivering 5-FU to tumor cells with greater efficiency than single ligand liposomal formulations in an *in vitro* brain tumor model. Therefore, is believed that a combination of Tf and penetratin have a synergistic effect in enhancing the uptake of liposomes across the BBB and that this may play key role in delivery of drug and induction of excellent anti-tumor efficacy in brain cancer cells [46].

Recent studies by Liu et al. reported that liposomes functionalized with Tf and arginine-rich residues as CPP sequences had a strong targeting efficacy on brain microvascular endothelial cell and brain glioma C6 cell uptake. This conferred a significant advantage for liposomal crossing across the BBB and entry into C6 glioma cells. Additionally, it has been shown that Tf-CPP decorated liposomes were able to

successfully escape from the endosomal compartment of C6 glioma cells to release the liposomal contents into the cytosol [47]. Recently, Zong et al. have developed dual-targeting doxorubicin liposomes (T7-TAT-liposmes) conjugated with cell-penetrating peptide (TAT) and peptide T7 (HAIYPRH), a unique targeting agent with high affinity for TfR, to transport drugs across the BBB, and to penetrate brain glioma. Their results indicated that T7-TAT-liposmes markedly enhanced *in vitro* cellular uptake and drug delivery compared with DOX liposomes. An *in vivo* study showed that T7-TAT-liposmes could cross the BBB and importantly penetrate the tumor and selectively deliver drug to glioma regions. Transport of liposomes across the BBB was markedly increased when they were decorated with both TAT and T7. Therefore, T7-TAT can act as an effective brain targeting ligand [48].

It has been reported that several receptors such as transferrin receptor, epidermal growth factor receptor insulin receptor, integrins and low-density lipoprotein receptor are overexpressed on brain tumor cells specially cancerous glioma cells [49]. Thus, dual targeting strategies could be used for the delivery of drugs specifically to brain tumors. Zong et al. used co-modified liposomes decorated with specific ligand T7 and nonspecific peptide TAT in order to enhance the BBB penetration, and then to increase the penetration efficiency in glioma tumor cells. *In vitro* cellular uptake in C6 and bEnd.3 cells and a BBB model indicated that the cellular uptake of T7-TAT- liposomes was significantly higher than those of T7-liposome, TAT-liposome and PEGylated liposomes. Furthermore, the hemolytic study showed that the outer PEG on the liposomal surface could shield TAT and reduce the hemolytic toxicity of the latter. Hence, the internalizing efficiancy of T7-TAT-liposomes demonstrates that the ligands T7 and TAT have a synergistic effect on the cellular uptake in a concentration-dependent manner and improve the cell penetration of liposomes. When T7 peptides are attached to the TfR, TAT peptide close to the surface of cell membrane they promote the cellular delivery liposomal cargo to the glioma cell. The *in vivo* biodistribution results showed that the accumulated of T7-TAT-liposome and the concentrations of doxorubicin in the brain was higher than all other liposomal formulations four hours after administration. Moreover, the hearts of the group treated with T7-TAT-liposomal loaded doxorubicin had lower concentrations of doxorubicin at four hours compared with other groups. Collectively, the above evidence indicates that T7-TAT-liposomal delivery system could effectively increase cellular uptake, transport across the brain, and enable the targeting of brain glioma tumor whilst minimizing the cardiotoxicity of doxorubicin [50].

Folate receptor-mediated transcytosis

Recently, liposomes modified with acid-cleavable (pH-sensitive) folic acid (FA) and dNP2 peptide have been used for the delivery of drug to the brain. dNP2 is a safe and humanized blood–brain barrier penetrating peptide [51]. FA may act by binding to the folate receptor (FR) on the BBB and enhancing transport across the BBB by receptor-mediated transcytosis [52]. Li et al. design paclitaxel (PTX) loaded liposomes co-modified with FA and dNP2 for efficient delivery to the brain metastasis caused by breast cancer. It is thought that the acid-cleavable FA drug-loaded liposomes accumulated at tumor site *via* the interaction of FA and folate receptor. The dNP2 peptide enhanced liposome uptake into tumor cells. Penetration studies using an *in vitro* BBB model indicated that the uptake of FA-dNP2 liposome by bEnd.3 cells was higher than single ligand modified liposomes (FA- liposome, dNP2 liposome). Therefore, FA and dNP2 have synergistic effect on the transportation across the bEnd.3 and were able to improve the delivery of PTX to orthotopic breast cancer and its metastatic sites in the brain [53]. In another study, Li et al. used PTX loaded liposomes co-modified with FA and dNP2 to improve the efficiency of penetration across the BBB and the targeting of glioma. The result indicated that co-modification PTX loaded liposome with FA and dNP2 has a synergistic effect on the targeting of FR-positive C6 cells. In addition, pH sensitive FA exhibited sensitive cleavage of FA at pH 6.8 and enhanced the effect of dNP2 and elevated the cellular uptake compared to non-cleavable FA and single modified liposomes. An *in vivo* study indicated that the dual modified liposomes displayed enhanced BBB transportation effects, greater accumulation in orthotropic glioma resulting in an improved therapy of tumors in a mouse model of glioma. The dual modified liposomes loaded with PTX had excellent penetration into tumor cells resulting in greater cytotoxicity and extended survival in these mice [54].

RGD modified liposome

The cell adhesion molecules including integrins are crucial for cell adhesion, migration, signalling and viability of most cells. These molecules are particularly overexpressed on cancer cells such as melanomas and glioblastoma. Thus, ligands that recognize specific integrin molecules are excellent candidates to target tumor cells [55]. In this regard, tripeptide motif arginine-glycine-aspartic acid (RGD) has been identified to have high affinity for integrins, particular for the αvβ3 integrin that is highly over-expressed on many cancer cells. To date, RGD sequence along with other molecules has been extensively used for targeted drug delivery to cancer cells, especially in brain tumor cells [55]. A study conducted by Qin et al. demonstrated that liposome modified by RGD and TF effectively target C6 and b.End.3 cell lines and significantly increased uptake and penetration into tumor cells. RGD/TF modified liposomes markedly increased the accumulation and distribution of liposomes in the brain *in vivo*. Additionally, PTX loaded

liposomes co-modified with RGD/TF more efficiently induced anti-proliferative activity against C6 cells and 3D tumor spheroids [56].

Belhadj et al. developed multi-functionalized liposome modified with cyclic RGD (c(RGDyK)) and p-Hydroxybenzoic Acid (pHA) to improve the efficiency of drug delivery and glioblastoma treatment. They used c(RGDyK) that could bind to integrin αvβ3 on the BBB and a small molecule ligand p-pHA which could bind to dopamine receptors (an attractive target, because of their abundant expression on the BBB) and increase cellular uptake through the pHA-dopamine special binding pathway. An *in vitro* study indicated that c(RGDyK)/pHA-liposomes could target glioblastoma cells and U87, bEnd.3 and HUVECs and increase cellular uptake efficiency. Furthermore, doxorubicin-loaded c(RGDyK)/pHA liposomes were able to penetrate into the tumor spheroids and increase the cytotoxicity of doxorubicin, thus inducing enhanced growth inhibitory effect on glioblastoma cells. *In vivo* work also demonstrated that the c(RGDyK)/pHA modified liposomes have a higher targeting ability and enhanced accumulation and distribution within the tumor resulting in a longer duration of survival than any other treatment groups Therefore, liposomes modification with c(RGDyK)/pHA enhanced anti-glioma efficacy drug such as doxorubicin for treatment of brain disorder through facilitate the accumulation and transferring more liposomes, hence showed significantly better anti-brain tumor effect in the tumor-bearing animal [57].

Peptide 22 (NH2-C6-(cMPRLRGC)c-NH2), is a specific ligand for Low-density lipoprotein receptors (LDLR) which are overexpressed on the BBB and glioma cells. Recently, Peptide 22 along with the ligand cRGD was used for the surface modification of liposomes (c(RGDfK)/Pep-22 liposome) and the ability of these liposomes were evaluated for facilitating drug delivery across BBB, BBTB and for their ability to target tumor cells and neovasculature. An *in vitro* study showed that cellular uptake of ligand decorated liposome c(RGDfK)/Pep-22 on BCECs, HUVECs and U87 cells was significantly higher than other prepared liposomes. The study further verified the importance of c(RGDfK)/Pep-22-liposomes for brain targeting and indicated that these liposomes accumulated to a greater extent in brain tumor tissue than single ligand modified liposomes. Therefore, it seems that c(RGDfK) and Peptide-22 have synergistic roles for the liposomal delivery across the BBB. Also, c(RGDfK)/Pep-22 liposome loaded with doxorubicin confers the longest median survival time in treated mice and inhibits the growth of glioma [58]. One of the major problems relating to the use of cRGD-modified nanocarriers is that these nanocarriers are mainly accumulated around the tumor site, rather than entering the tumor parenchyma [59]. To improve the BBB penetration of cRGD-modified nanocarriers across the BBB and into the tumor

parenchyma, Shi et al. used a multifunctional peptide TR, a tandem peptide consisting of cRGD and histidine-rich TH peptide. TH peptide possesses the capacity of 'proton sponge effect' and pH-responsive cell penetration, hence was able to enhance nanoparticle penetration into the core of tumor. Hence, cRGD-modified nanocarriers were able to target the integrin αvβ3 and also, increase the ability of nanocarrier penetration at tumor sites [60]. Shi et al. used liposomes modified by TR peptide to enhance the transport efficacy across the BBB. They indicated that PTX-loaded liposomes modified with TR peptide have a very high affinity for integrin αvβ3 and improved BBB penetration and therapeutic efficacy in a glioma model. Therefore, it seems that TR peptide plays a key role in the transportation of PTX-loaded liposome to the brain. An *in vitro* study has shown that PTX-TR-liposome exhibited the greatest anti proliferative effects against C6 glioma cells and brain cancer stem cells (CSCs) when compared with PEG- and RGD-modified liposomes. Also, this formulation was able to effectively destroy the glioma vasculogenic mimicry (VM) channels [60].

Glucose mediated transporter

Glucose transporter 1 (GLUT1) is one of the major carrier-mediated transporter system that is abundant on the surface of endothelial cells and glioma cells in the brain. GLUT1 is responsible for transporting glucose from the blood into the extracellular space of the brain. Glucose is an essential nutritional substance for brain function but could be exploited as a carrier for brain targeting drugs. GLUT1 is therefore a promising and efficient transportation carrier to facilitate the delivery of drugs to the brain [61]. Recently, liposomes modified with glucose have been for this purpose [61, 62]. For example, Xie et al. demonstrated that PEGylated liposomes modified by glucose possess the potential of brain targeting and exhibited an enhanced efficiency for brain delivery [63]. In another study, Qin et al. used a glucose-mediated liposome as a brain delivery system. Their data indicated that glucose-mediated liposomes were able to transport drugs across the BBB and that this approach significantly enhanced drug accumulation in the brain [64]. In a recent investigation, Peng et al. developed a novel dual brain-targeting glucose-vitamin C (Glu-Vc) modified liposome to enable the efficient delivery of paclitaxel (PTX) to the brain. A cellular uptake assay on GLUT1- and SVCT2-overexpressed C6 cells indicated that Glu-Vc-liposome have a higher rate of uptake in comparison to unmodified and singly-modified liposomes. Also, the Glu-Vc modified liposomes showed higher targeting ability *in vivo* and exhibited maximum accumulation of drug-loaded liposomes at tumor sites [65]. Recent evidence suggests that substances with similar structures to glucose including 2-deoxy glucose, galactose, mannose, and glucose analogs are able to pass through the BBB *via* glucose mediated transporters [66]. Because of the affinity

of GLUTs for mannose, liposome decorated with mannose derivates have been used as a recognition marker for brain targeting and studies have indicated that mannose modification of liposomes plays a major role in the transport of liposomes across the BBB [67-69]. Previous work conducted by Hao et al. demonstrated that P-aminophenyl-α-d-mannopyranoside (MAN) modification of liposomes was able to cellular uptake in C6 glioma cells *in vitro* and to promote penetration through the BBB into brain and accumulation in the intracerebral regions such as cerebellum and cerebral cortex [70]. Later, Du et al. found that MAN-modified liposome may enter the brain through GLUT1 and GLUT3 transporter pathway. They showed that MAN may mediate the transport of the MAN modified liposomes across BBB through GLUT1 and GLUT3 [71]. Moreover, Ying et al. developed dual-targeting daunorubicin-loaded liposomes by conjugating with MAN and TF to improve the transport of drug across the BBB and into glioma. MAN-TF targeting daunorubicin liposomes significantly increased cellular uptake by C6 glioma cells and exhibited the strongest dual-targeting effects and transportation efficacy across the BBB model compared with non-targeted liposomes and liposomes targeted with either MAN or TF. Also, an *in vivo* study showed that tumor-bearing rats treated with dual-targeting daunorubicin liposomes have a higher median survival time and were able to evidently reduce the volume of tumor competed to free daunorubicin and other control groups [69]. It has also been reported that liposomes which had been modified with MAV and cell penetrating peptides such as penetratin (Pen) or rabies virus glycoprotein (RGV) on the surface, promote selective and enhanced delivery to the brain [72, 73]. Based on the reported studies and the rationale for using GLUT1 targeting ligands for brain-targeted delivery of nanoparticles, it seem that liposomes modified with glucose and MAN are promising vehicles for delivery of cargoes to the brain.

Immunoliposomes

Surface functionalization of liposomes by antibody (immunoliposomes) is an exciting potential approach to allow targeted delivery of drugs and diagnostic agents to specific tissues [74]. OX26 and RI7217 are a well-known monoclonal antibody (mAb) with high affinity for rat and mouse transferrin receptor respectively and are able to cross the BBB by transferrin receptor-mediated transcytosis [75, 76]. Huwyler et al. developed PEG-liposomes conjugated with OX26 mAb for targeted drug delivery to brain. They indicated that OX26 PEGylated liposomes are capable of successfully transferring daunomycin into the rat brain [74]. Recently the effect of OX26 immunoliposomes were investigated for their ability to bind to BCECs and thereby to transport substances to the brain. This study demonstrated that OX26

decorated liposomes enhanced the ability of binding to BCECs through an active endocytotic uptake mechanism and increase immunoliposome accumulation in the BCECs of the BBB [77]. Kong and colleagues used PEGylated liposomes conjugated with OX26 mAb as carriers of dopamine in animal model of Parkinson's disease (PD). They indicated that the uptake of dopamine-loaded PEGylated OX26-immunoliposome in the brain in a rat model of PD is higher than encapsulated dopamine–PEGylated liposomes and dopamine alone. It was also demonstrated that the brain distribution of PEGylated OX26-immunoliposome was significantly greater than dopamine–PEGylated liposomes which is due to the effective role of OX26 mAb in binding to the transferrin receptor of the brain capillary endothelium that leading to increased efficient and specific delivery of liposome to brain tissue [78]. Dual PEGylated immunoliposomes, composed of OX26 and anti-α-synuclein LB509 antibodies, were developed by Loureiro et al. to enhanced drug delivery to brain in PD. The study indicated that these immunoliposomes were able to target the BBB trough TF receptors and α-synuclein protein (aneuronal protein that is assosiated with Parkinson's disease) and effectively enhanced the transport of drugs across the BBB [79]. Recently, Gregori et al. employed a novel approach by using MYBE/4C1 antihuman TfR mAb for the surface functionalization of liposomes. They demonstrated that functionalization with MYBE/4C1 mAb improved the passage of doxorubicin-loaded liposomes in an *in vitro* BBB model [80]. CD133, is a 120 kDa transmembrane single-chain transmembrane glycoprotein which is expressed in cancer stem cells such as glioblastoma stem cells (GSCs) [81]. Recently, immunoliposomes modified with CD133 have been used as a targeting ligand to GSC. In this study, dual-modified immunoliposomes conjugating with angiopep-2 and CD133 antibody were used for the targeting of GSC [82]. Angiopep-2 (TFFYGGSRGKRNNFKTEEY) is a peptide derivative of the Kunitz domain with good BBB penetration. Angiopep-2 extensively used to target the low-density lipoprotein receptor related protein 1 (LRP1) which is expressed both in the BBB and on glioblastoma cells [83]. Kim et al. indicated that dual targeting immunoliposome modified by angiopep-2 and CD133 loaded with temozolomide (TMZ) (Dual-LP-TMZ) increased cytotoxicity and apoptosis against U87MG GSCs *in vitro* compared to free TMZ and non-targeted liposomes. *In vitro* experiments indicated that the mice treated with Dual-LP-TMZ exhibited lower tumor size, and highest median survival time (MST) and increased life span (ILS) compared to free TMZ and non-targeted liposomes [82]. In summary, the available evidence demonstrates that that antibody as a specific targeting ligand provides a high targeting affinity with receptors and significantly enhances the efficiency of drug delivery to the brain.

Cationic liposomes

In recent years, cationic liposomes have been developed as a potential brain drug delivery vehicle. This type of liposome is negatively charged at physiological pH. Therefore, these liposomes are able to attach to the molecules that are positively charged at physiological pH *via* electrostatic interaction [84]. Chen and colleagues, developed a lactoferrin-modified procationic liposome as a potential brain drug delivery vector. They used Cholest-5-en-3-ol-(3)-(2-((4-((carboxymethyl) dithio]-1- iminobutyl) amino) ethyl) carbamate (CHETA, C36H61N3O4S2), (a cholesterol derivative), to prepare the procationic liposomes [85, 86]. Lactoferrin which is a cationic iron-binding glycoprotein belonging to the transferrin family was used as a targeting ligand for delivery of drug to the brain. Lactoferrin was able to attach to the lactoferrin receptor, which is highly expressed on the surface of brain endothelial cells. Receptor-mediated transcytosis across the BBB was thereby enhanced [87]. The cationic liposomes modified with lactoferrin confer two important features on these delivery systems. First, lactoferrin has a positive charge at physiological pH, therefore, is able to be easily absorbed onto the negatively charged surface of the procationic liposome *via* electrostatic interaction. Secondly, high-affinity binding of lactoferrin to the lactoferrin receptors on brain cells leads to improved delivery of drug to the brain [86, 88]. The experiments conducted by Chen et al. indicated that procationic liposome modified with lactoferrin served as brain specific targeting ligands and showed improved performance in the uptake efficiency and cytotoxicity in primary brain capillary endothelial cells. They also have a greater ability to cross BBB *in vitro* compared to conventional and cationic liposomes [85]. In another study, Chen et al. studied the therapeutic effects of doxorubicin-loaded procationic liposomes for glioma treatments. Their results show that these modified liposomes improved the uptake efficiency in BCECs and C6 cells and could effectively inhibit the growth of C6 *in vitro*. In *in vivo* models, survival time was longer compared with other DOX formulations [89]. Moreover, several studies have demonstrated that fusogenic liposomes composed of pH sensitive and cationic liposomes (such as neutral lipid dioleoylphosphatidylethanolamine (DOPE) combined with the cationic lipid 1, 2-dioleoyl-3-trimethylammoniumpropane (DOTAP)) enhance cellular cytoplasmic delivery [90, 91]. Recently, it has been demonstrated that fusogenic liposomes effectively enhance cytoplasmic delivery of their cargos to bEnd.3 cells [73]. Therefore, it seems that the presence of cationic lipid in liposomal formulations improves cellular cytoplasmic delivery by inducing membrane fusion via electrostatic interactions with the cell membranes.

Future perspectives

Liposome-based strategies are one of the most promising approaches to facilitate the delivery of drugs to the brain. To date, a number of studies have been performed using liposomal carrier systems, but many of them have so far been limited to preclinical studies extensive further investigation, particularly for toxicity , is necessary prior to clinical use, to enable this technique to be widely employed in a range of CNS and brain disorders. In addition, the clinical success of liposomal therapies will require an interdisciplinary group of researchers with expertise in liposome technology, neuroscience, oncology, pharmacology and medical imaging. The nanoliposomes need to be less than 100 nm in diameter to enable them to cross the BBB deliver drug to the brain. Many of drugs that are used in the treatment of brain cancer (including glioblastoma) are highly cytotoxic. In order to reduce the toxicity of these agents, they must be specifically targeted to the affected site to overcome the side effects of non-specific binding. Therefore, the formulation of nanoparticles must be optimized to meet these needs. Extensive research investment in this field is justified by the high market price that successful agents would attract.

Furthermore, most of the research has been conducted on brain tumors, and reports on other CNS disorders arerelatively rare, and require further investigation. However, a major limitation of current liposomal brain cancer therapies is the low ability and inhomogeneous distribution of liposome therapeutics to penetrate the BBB, to accumulate in the tumor region andto enter the tumor mass. This problem is not unique to liposomal drug delivery to the brain, but is a common problem limiting the effectiveness of all types of therapeutic agents , including other nanoparticle-based drug delivery systems. Therefore, different strategies should be considered to improve the intratumoral distribution of liposome therapeutics. One problem is the rapid clearance from the circulation by the reticuloendothelial system (RES) organs, an issue which has been partially resolved by modification of the size and shape of particles and pegylation of the liposomal formulation. Furthermore, targeted drug delivery by specific ligands offers a significant advantage by promoting more efficient delivery of therapeutic compounds to specific cells or tissue of the body and minimizing the exposure of non-target tissues to the drug. Additionally, the results of several studies suggest that intratumoral administration can be increase tumor liposome concentrations and improve the accumulation and distribution of liposomes within the tumor. Increased understanding of the BBB, the blood-cerebrospinal fluid barrier, the mechanisms of drug movement within the CNS, tumor biology and macromolecular structure and nanoparticle transport properties, may lead to advances in technology, and further therapeutic gains for drug delivery to the brain in the near future.

Conclusions

The treatment of central nervous system (CNS) disorders remains challenging due to the functions of the BBB, which impedes the delivery of many therapeutic drugs to the brain. Therefore, development of novel therapeutic strategies for drug delivery to the brain tissue and treatment of neurological disorders is a major prerequisite for the clinical application of many drugs. The use of nanotechnology-based drug delivery systems such as liposomes has great potential to improve the therapy of a range of neurological disorders. Liposomes are promising carriers for drug delivery to the CNS and offer various advantages for drug delivery over other nanocarrier systems since they are easy to prepare and are highly biodegradable and biocompatible. Moreover, liposomes can minimize the side effects of drugs, decrease required drug dose, increase drug half-life, enable controlled drug release and enhance penetration across the BBB. Moreover, passive or active targeting of drugs to brain regions is achievable using surface modification of liposomes and by creating liposomes covalently coupled with specific ligands (such as TF, FA) and coating their surface with certain hydrophilic polymers such as PEG (**Table 1**). A wide variety of liposomal formulations with a range of structural modifications and features have been used to enhance the delivery of drugs to the CNS. Such approaches are extremely promising, however at present the quantity of drug that can be delivered to the brain by these mechanisms is small in comparison with the delivery of free (non-liposomal) drugs to other organs and tissues. Extensive work is required to improve our understanding of the mechanisms which manage the transportation of drug loaded liposomes to the brain and to investigate the clinical efficacy and safety of these preparations in patients.

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Figure Legend

Figure 1. A) Structure of the neurovascular unit. Pericytes surround brain microvascular endothelial cells and together with the basal lamina and astrocytic end-feet, they contribute to the organization of the BBB and form the neurovascular unit. B) Schematic representation of the different mechanisms of transport of molecules across the blood-brain barrier. Paracellular pathway: very small hydrophilic molecules penetrate the BBB through the tight junctions. Transcellular pathway (diffusion): small lipophilic molecules can diffuse across the endothelial cells passively. Transport proteins pathway: specific molecules such as amino acids, glucose and nucleosides could be non-covalently binding to the protein transporters on one side of the membrane and released on the other side. Receptor-mediated transcytosis: larger molecules such as insulin, transferrin and low-density lipoprotein (LDL) are transported through specific receptors. Adsorptive mediated transcytosis: cationic drug could be electrostatically attracted anionic sites present on the cell membrane and increases its uptake by adsorptive mediated transcytosis or endocytosis. Efflux Pumps: these pumps are responsible for drug expulsion from the brain.

Table 1. Effects of different liposomal preparations in penetrating the brain tissue.

Surface modification	Study type	*In vitro* Cell type	Targeting ligand	Delivered Drug	Function	Ref.
PEGylation	*In vivo*	-	-	Resveratrol	PEGylated liposomes have a longer systemic circulation time and more extensive accumulation in the brain.	[19]
	In vitro	bEnd.3[1] /astrocytes C8D1A cells	-	Riluzole	More extensive accumulation of the drug in the brain.	[22]
	In vivo					
TPGS coating	*In vitro*/ *In vivo*	C6 glioma cell	-	Resveratrol	TPGS coated liposomes have excellent cellular internalization, and more extensive accumulation in brain.	[20]
	In vitro	C6 glioma cell	-	Docetaxel	More extensive cellular uptake and cytotoxicity in C6 glioma cells.	[21]
CPP	*In vitro*/ *In vivo*	BCECs/ C6 glioma cells	TAT	Doxorubicin	More extensive cellular uptake and accumulation of drug in brain, Less cardiotoxicity.	[28]
	In vitro/ *In vivo*	U87MG cells/ HUVECs	iNGR	Doxorubicin	More extensive cellular uptake and accumulation of the drug in the brain, Increased survival time in an animal model.	[32]
TF	*In vitro*/ *In vivo*	U87 glioblastoma cell line	TF[2]	Resveratrol	More extensive cellular uptake/ induced significantly greater apoptosis and cell cycle arrest and accumulation of drug in tumor. Increased survival time in an animal model.	[38]
	In vitro	hCMEC/D3	TF	Dopamine	successfully transferred across *in vitro* model of the BBB	[39]
	In vitro/ *In vivo*	C6 cell	TF	Vincristine/ Tetrandrine	More extensive cellular uptake/ inhibiting the cancer cell invasion and VM channels/ more extensive accumulate in brain tumor site	[40]
Tf-CPP	*In vitro*	U87 glioblastoma cell line	Tf- PFVYLI	Doxorubicin and Erlotinib	More extensive cellular uptake, incurring drug concentration in tumor cells inside, enhanced tumor cell death and antitumor efficacy in glioblastoma tumor cells	[43]
	In vitro	Primary glial cell, bEnd.3	Tf- PR[3]	No	More extensive cellular uptake and transfection, improved cell penetration	[42]
	In vitro/ *In vivo*	Daoy medulloblastoma, U87 glioblastoma, bEnd.3	Tf-TAT, Tf-Penetratin, Tf-Mastoparan	Doxorubicin	Tf-Penetratin liposomes have an efficient cellular uptake, more extensive translocation of doxorubicin,	[45]
	In vitro/ *In vivo*	U87 glioblastoma, bEnd.3	TF-penetratin	5-fluorouracil	More extensive cellular uptake, induced significantly more extensive apoptosis in U87 cell. Induction of excellent anti-tumor efficacy in brain cancer cells.	[46]

	In vitro	C6 glioma cell	TF-arginine-rich residues	No	More extensive cellular uptake, successful escape from endosomal compartment of glioma C6 cells	[47]
	In vitro/ *In vivo*	C6 glioma cell, bEnd.3	T7 peptide[4]-TAT	Doxorubicin	More extensive cellular uptake, Increased brain targeting efficacy	[48]
	In vitro/ *In vivo*	U87, bEnd.3 and glial cells	TF-QLPVM, TF-TAT	Doxorubicin, erlotinib	More extensive cellular uptake, Increased brain targeting efficacy	[44]
	In vitro/ *In vivo*	bEn.d3, **p**rimary neuronal, glial cells	PFVYLI, R9F2	pDNA	Enhanced *in vitro* transfection efficacy, superior ability to translocate *in vitro* and *in vivo* BBB	[92]
FA-CPP	*In vitro*/ *In vivo*	bEnd.3 cells	FA[5]-dNP2[6]	Paclitaxel	Enhanced BBB transportation effect, more extensive accumulation of drug in tumor cells	[53]
	In vitro/ *In vivo*	C6 glioma cell	FA-dNP2	Paclitaxel	Enhanced BBB transportation effect, more extensive accumulation of drug in tumor cell.	[54]
RGD	*In vitro*/ *In vivo*	C6, b.End.3 cell	TF-RGD[7]	Paclitaxel	More extensive cellular uptake, highest brain distribution,	[56]
	In vitro/ *In vivo*	U87, b.End.3 cell, HUVECs[8]	c(RGDyK)/pHA[9]	Doxorubicin	More extensive cellular uptake, increased cytotoxicity of doxorubicin and induced the strongest inhibitory effect on glioblastoma cell growth *in vitro* and *in vivo*	[57]
	In vitro/ *In vivo*	BCECs[10], HUVECs and U87 cells	c(RGDfK)/Pep-22	Doxorubicin	More extensive cellular uptake *in vitro*, more extensive distribution in brain tumor, longest median survival time in treated mice and inhibiting growth of glioma	[58]
	In vitro/ *In vivo*	C6 glioma cells	cRGD and histidine-rich TH peptide	paclitaxe	Greater affinity for integrin αvβ3, more extensive abilities for transferring liposomes across the BBB, improving therapeutic efficacy in brain glioma-bearing animals	[60]
Glucose	*In vitro*/ *In vivo*	BCECs	Glucose	No	Greater potential for brain targeting and strongest brain delivery efficacy.	[63]
	In vitro/ *In vivo*	BCECs	Glucose	No	Greater potential for transport of drug across the BBB, increased accumulation of drug in the brain	[64]
	In vitro/ *In vivo*	C6 cells	Glucose-vitamin C	Paclitaxel	Greater *in vivo* targeting ability, exhibiting maximum accumulation of drug-loaded liposomes at tumor sites	[61]
	In vitro/ *In vivo*	C6 glioma cells	MAN[11]	No	Greater cellular uptake, promoting penetration through the BBB into the brain, accumulation in the intracerebral regions	[70, 71]
	In vitro/ *In vivo*	C6 glioma cells	MAN/ TF	Daunorubicin	More extensive cellular uptake, greater median survival time	[69]

	In vitro/ *In vivo*	b.End.3 cell	MAN- RGV[12], MAN-Pen[13]	ApoE2 encoding plasmid DNA (pApoE2)	Improved transport and transfection of ApoE2 gene across the *in vitro* and *in vivo* BBB model	[73]
	In vitro/ *In vivo*	b.End.3 cell, primary glial, primary neuronal cells	MAN-RGV, MAN-Pen	BDNF[14] encoding plasmid (pBDNF)	Higher transfection efficacy, more extensive cellular uptake	[72]
Immunoliposome	*In vivo*	-	OX26 mAb	Daunomycin	Delivery of daunomycin to the rat brain	[74]
	In vitro	BCECs	OX26 mAb	No	Enhanced ability of binding to BCECs, increasing immunoliposome accumulation in the BCECs	[77]
	In vivo	-	OX26 mAb	Dopamine	Increased cellular uptake, increased delivery of liposome to brain tissue	[78]
	In vitro	hCMEC/D3	OX26-LB509 Ab	EGCG[15]	Increased transport of drugs across the BBB	[79]
	In vitro	hCMEC/D3	MYBE/4C1	Doxorubicin	Increased passage of doxorubicin-loaded liposome cross an *in vitro* BBB model	[80]
	In vitro/ *In vivo*	U87	CD133- angiopep-2	Temozolomide	Increased *in vitro* cytotoxicity and apoptosis, Exhibiting smaller tumor size, and higher median survival time	[82]
	In vitro/ *In vivo*	hCMEC/D3, U87-MG cells	R17217 mAb	Docetaxel	Enhanced *in* vitro cellular uptake, increased *in vitro* penetration across BBB model, improved in vivo brain targeting, enhanced the efficacy of drug delivery in animal model	[76]
Cationic liposome	*In vitro*/ *In vivo*	BCECs, C6	lactoferrin	Doxorubicin	Improved cellular uptake, inhibiting the growth of C6 *in vitro* and enhancing survival time *in vivo* animal models	[85, 89]
	In vitro	b.End.3 cell	MAN/ RGV/Pen	No	Fusogenic liposomes enhanced cytoplasmic delivery of cargos and reduced endocytosis	[73]

[1] brain capillary endothelial cells (bEnd.3), [2]transferrin (Tf), [3] poly-L-arginine (PR), [4] T7 peptide (HAIYPRH), [5]folic acid (FA), [6] a safe and humanized blood–brain barrier penetrating peptide, [7]arginine-glycine-aspartic acid (RGD), [8]human umbilical vein endothelial cells (HUVECs), [9] p-Hydroxybenzoic Acid (pHA), [10]brain capillary endothelial cells, [11]P-aminophenyl-α-d-mannopyranoside (MAN), [12]rabies virus glycoprotein peptide (RGV), [13]penetratin (Pen), [14] Brain-derived neurotrophic factor (BDNF), [15]flavonoid epigallocatechin-3-gallate (EGCG),