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# Nanoparticle designs for delivery of nucleic acid therapeutics as brain cancer therapies

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## Abstract

Glioblastoma (GBM) is an aggressive central nervous system cancer with a dismal prognosis. The standard of care involves surgical resection followed by radiotherapy and chemotherapy, but five-year survival is only 5.6% despite these measures. Novel therapeutic approaches, such as immunotherapies, targeted therapies, and gene therapies, have been explored to attempt to extend survival for patients. Nanoparticles have been receiving increasing attention as promising vehicles for non-viral nucleic acid delivery in the context of GBM, though delivery is often limited by low blood-brain barrier permeability, particle instability, and low trafficking to target brain structures and cells. In this review, nanoparticle design considerations and new advances to overcome nucleic acid delivery challenges to treat brain cancer are summarized and discussed.

### Keywords

Nanoparticles; Biomaterials; Gene delivery; RNA delivery; BBB crossing; Targeted delivery; Glioblastoma; Intracellular delivery

## 1 Introduction

There are over 100 types of primary central nervous system (CNS) cancers with distinct histological features [1]. Malignant tumors comprise 30.9% of these cancers, with glioblastoma (GBM) as the most common of these malignancies (47.7%), associated with a five-year survival rate of only 5.6% [1]. The dismal prognosis associated with GBM has fueled extensive research into therapies for patients, leveraging and combining a variety of approaches such as immunotherapy, targeted therapy, and gene therapy. This review gives an

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Conflicts of Interest

Patents related to technology discussed in the manuscript have been filed by Johns Hopkins University with co-inventors J.K., K.M.L., S.Y.T., and J.J.G. J.J.G. is a co-founder, manager and CTO of Dome Therapeutics and is a scientific advisory board member of Tidal Therapeutics. Any potential conflicts of interest are managed by the Johns Hopkins University Committee on Outside Interests.

overview of technological advances with a particular focus on therapeutic nanoparticle gene delivery to GBM.

A hallmark of GBM is extreme heterogeneity, both inter- and intratumorally, which decreases tumor-targeting ability and adds complexity to therapeutic design [2]. In 2016, the World Health Organization (WHO) Classification for GBM was updated to include the key molecular marker isocitrate dehydrogenase (IDH) [3], with IDH mutants behaving distinctly from wild-type GBM. Meta-analysis of IDH-1/2 mutational status has indicated improved overall survival and progression-free survival in patients carrying mutations, though the specific biological role of the IDH-1/2 mutation requires further investigation [4]. Additional markers, such as NF-1 and EGFRvIII, though not included in the WHO classification, have been explored with the aim of understanding additional GBM subgroups with impactful clinical correlations [5].

### 1.1 Standard of Care

The standard of care for GBM focuses on surgical resection, utilizing intraoperative image guidance if available to achieve maximal safe resection [6]. Surgeons must consider the extent of resection carefully, balancing the need for aggressive tumor removal with the risk of patients' experiencing permanent deficits following surgery [7]. The importance of achieving gross total resection (GTR) is highlighted by its correlation with maximized overall survival, regardless of whether GTR is achieved in a primary surgery or in a subsequent surgery following recurrence [8]. Additional techniques, such as preoperative imaging as well as intraoperative image guidance and language and motor mapping, have been shown to increase the likelihood of maximal safe resection in GBM patients [9]. Biodegradable carmustine wafers (Gliadel® Wafers) may also be implanted during primary surgical resection to deliver the chemotherapeutic locally to the tumor site, and they have shown benefit to GBM patients [10], highlighting the utility of local administration of a therapeutic during surgery.

The GBM standard of care also involves radiotherapy following surgical resection, with dosage and fractionation schedules carefully studied to deliver maximum benefit to patients while considering potential risks associated with the treatment [11]. Radiotherapy has also been accompanied by dosing of the chemotherapeutic agent temozolomide (TMZ) since studies show combination therapy offers a significant survival benefit compared to radiotherapy alone [12]. Following tumor resection, TMZ can be combined with tumor-treating fields, in which low-intensity alternating fields are applied at the scalp. This combination has shown significant improvement in progression-free survival and overall survival compared to TMZ alone [13]. Other systemic therapies, such as lomustine, vincristine, carmustine, and procarbazine may also be used with tumor recurrence, though data on these treatment regimens are sparse, and no true standard of care exists for instances of recurrence [6]. Despite surgical intervention, radiotherapy, and systemic chemotherapies, supportive care becomes a critical component of care with disease progression. Antiepileptic drugs, corticosteroids, sedatives, and analgesics may be administered to support patients through palliative care [14].

## 1.2 Therapeutic Approaches

**1.2.1 Nucleic Acid Therapeutics—**The nanoparticles discussed in this review are engineered for delivery of DNA or RNA therapeutics, including small interfering RNA (siRNA), microRNA (miRNA), and messenger RNA (mRNA). The technology of using nucleic acid-based therapeutics is powerful, as it enables precise modulation of the expression of genes known to be involved in disease progression and can thereby be used for precision medicine (Table 2). DNA and mRNA are used to induce a specific gene of interest [15], whereas siRNA and miRNA are used for silencing of specific genes.

The sequence of nucleic acids can further easily be modified to enable patient-specific treatments and can encode essentially any gene involved in specific molecular pathways or oncogenes to facilitate treatments of otherwise so-called “undruggable” tumors [16]. Accordingly, nucleic acid therapeutics can be designed to target specific genes involved in the proliferation, migration, invasion, apoptosis, and angiogenesis of the malignant glioma cells, including gene editing correction using CRISPR-Cas9 [17]. Alternatively, they can also be used to enable cancer immunotherapies by inhibiting immune-suppressive genes expressed in brain tumors, reprogramming the tumor microenvironment to become pro-inflammatory, or inducing immune responses against cancer-specific antigens as nucleic acid-based cancer vaccines [15].

**1.2.2 Suicide Gene Therapy—**Suicide gene therapy, in which cancer cells are reprogrammed with genes that will lead to targeted apoptosis upon systemic administration of a cytotoxic prodrug, has been explored in the context of GBM. A commonly studied system for suicide gene therapy involves inducing the expression of herpes simplex virus thymidine kinase (HSV-tk) in cancer cells, which converts an otherwise non-toxic prodrug ganciclovir into a toxic substrate [18]. This leads to cell death in HSV-tk-expressing cells with a significant bystander effect. Despite promise in animal models, a phase III clinical trial comparing GBM patients treated with or without HSV-tk/ganciclovir retroviral suicide gene therapy following surgical resection and radiotherapy indicated no improvement in progression or overall survival [19]. Limitations noted in the study, however, suggest that suicide gene therapy for GBM could be more viable with increased transfection efficiency of target genes as well as improved delivery of the gene delivery vectors, topics that are addressed in depth in this review. Furthermore, exploration of gene delivery vectors beyond viral vectors also holds immense promise in expanding the utility of suicide gene therapy for GBM.

**1.2.3 Immunotherapy—**As the field of cancer immunotherapy continues to grow, there is much interest in leveraging the immune system against GBM. The brain had previously been thought to be an immune-privileged region, however more recent research has refuted this idea with the discovery of CNS lymphatics and the presence of immune cells in the CNS [20,21]. Despite immunological activity in the brain, GBM represents a tough hurdle for the immune system, as the cancer cells promote a highly immunosuppressive microenvironment, secreting immunosuppressive cytokines, modulating antigen presentation by tumor cells, and directly inhibiting T-cell function through immune checkpoints [22,23]. Preusser *et al.* discuss recent advances with checkpoint inhibitors for GBM focusing on clinical data

and highlighting key challenges that remain for GBM immunotherapies [24]. Gene-delivery approaches can be used to enhance immunotherapy to treat brain cancer as highlighted below.

Vaccine strategies have also explored for use in GBM. Cancer vaccines educate the adaptive immune system to recognize antigens expressed by tumors, ultimately leading to immune destruction of cancer cells. A 2019 phase 1b study of a neoantigen vaccine demonstrated increased T-cell infiltration in GBM patients following surgical resection and radiotherapy, indicating that personalized therapeutics hold promise in being able to productively influence a patient's immune environment [25]. Vaccines targeting common mutations, such as EGFRvIII, which is expressed in over 50% of GBM cases, have also been explored and demonstrated an increase in overall survival, though loss of expression was seen in 82% of patients upon recurrence [26]. These results suggest that cancer vaccines for GBM may require multiple targets as resistance mechanisms arise. Cuoco *et al.* provide a comprehensive review on cancer vaccines against GBM [22], and recent progress on the use of gene therapy to improve cancer vaccines are discussed in greater detail in later sections of this review.

Additional immunotherapy modalities have also been explored for GBM, including oncolytic viral therapies and chimeric antigen receptor (CAR) T-cell therapy. Oncolytic viruses, which selectively replicate in and lyse tumor cells, have been used in several clinical trials, and while they have demonstrated safety, they have so far shown a general lack of efficacy in patients [27]. CAR T-cell therapies also hold promise for treatment of GBM. CAR T cells are engineered to be specific for a particular antigen of interest and contain intracellular signaling domains that give rise to T-cell activation upon antigen recognition. One GBM patient received an intracranial infusion of CAR T cells and exhibited tumor regression with associated increases in immunostimulatory cytokines and immune cell population [28]. Another study demonstrated that adaptive resistance mechanisms arose following administration of CAR T cells to GBM patients, suggesting that such therapies may require dosing in combination with other therapeutics to address the increasingly immune-suppressive environment [29]. Overall, immunotherapy holds great promise for future GBM therapeutics, and Patel *et al.* provide a comprehensive review and perspective on incorporating immunotherapies into GBM treatment in conjunction with current standard of care practices [30]. Key to the development of this technology is safe and effective gene delivery to T cells, and novel methods to achieve efficient transfection of immune cells using nanoparticles are discussed in detail in this review.

#### 1.2.4 Small Molecules and Antibodies Targeting Cancer-Promoting Pathways

—As genetic and epigenetic screening capabilities have increased, molecular targets associated with GBM have been revealed. Tyrosine kinase receptors, such as epidermal growth actor receptor (EGFR), phosphoinositide 3-kinase (P13K), fibroblast growth factor receptor (FGFR), the proto-oncogene BRAF, and others, are often highly expressed and/or mutated in GBM tumors [31]. Additional targets identified in the tumor microenvironment, such as vascular endothelial growth factor (VEGF) and programmed death 1 (PD-1), are also attractive candidates for targeted therapies [31]. Many of these targets have the potential to be addressed via small molecule inhibitors or monoclonal antibodies, as reviewed by

Taylor *et al.* [32]. Various other systemic therapies have also been explored—for example, bevacizumab, an antiangiogenic monoclonal antibody, had an effect on progression-free survival, though it was associated with a higher rate of adverse events and did not change overall survival [33,34]. Overall, GBM tumor heterogeneity and development of resistance mechanisms suggest that targeted therapies are a challenging approach for the treatment of GBM, and may be best suited in combination with other therapeutics. Some of the molecular pathways of interest may also be more easily targeted by nucleic acid-based therapies, rather than traditional small-molecule drugs and monoclonal antibodies, and examples of these are elaborated on below.

### 1.3 Gene Delivery Systems and Routes of Administration

Viral vectors have so far dominated the field of GBM therapeutics and are notable for high transfection efficacy, making them advantageous for facilitating efficient gene transfer. Common viral vectors utilized in the context of GBM include retroviruses, adenoviruses, adeno-associated viruses, and lentiviruses, as reviewed by Manikandan et al. [35]. Mozhei *et al.* provide a comprehensive review of GBM clinical trials involving viral vectors, as well as perspective on key challenges associated with viral gene therapy [36].

This review focuses primarily on recent classes of non-viral vectors that have received increased attention due to their safety, efficacy, and ease of further modification. Non-viral vectors are advantageous for their lower immunogenicity compared to viral vectors and are often more adaptable, which can be used to improve their cellular targeting. Key categories of non-viral vectors include dendrimers, liposomes (including lipoplexes), lipid nanoparticles (LNPs), polymeric nanoparticles (including polyplexes), and spherical nucleic acids (SNA). Many polymeric and lipid-based formulations rely largely or in part on electrostatic interactions between a cationic biomaterial and the anionic nucleic acid cargo, causing self-assembly into dynamic nanoscale electrostatic complexes termed polyplexes or lipoplexes. Other solid nanoparticles are based on insoluble polymers or metals, and nucleic acids can be loaded by absorptive processes or chemical conjugation. Synthetic, non-viral particles can be flexibly designed to have the desired physical, chemical, and biological properties for effective gene delivery as discussed at length throughout this review.

Substantial work has also been conducted to improve routes of delivery for GBM therapeutics. Systemic delivery, while advantageous for ease of administration, is largely limited by a therapeutic's ability to cross the blood-brain barrier (BBB), making it difficult to achieve a therapeutic dose in relevant areas of the brain. Local delivery, often performed during surgical procedures, obviating the need for an additional invasive event, allows more direct interaction with critical structures. One method of local delivery, convection-enhanced delivery (CED), uses an implanted catheter for delivery to the brain, utilizing positive pressure to increase distribution of a therapeutic by convection rather than relying on diffusion alone, but this requires invasive measures. Convection-enhanced, intratumoral delivery of a recombinant poliovirus demonstrated a higher survival rate of patients with recurrent glioblastoma after 24 and 36 months illustrating the utility of CED to the brain [37], and Jahangiri *et al.* provide a comprehensive review of CED for glioblastoma in preclinical and clinical development [38]. Key hurdles associated with therapeutic design

and delivery include BBB permeability, formulation stability, cancer cell targeting and specificity, cellular uptake, and safety considerations (Figure 1). Multiple methods have been explored to help nanoparticles overcome such hurdles as discussed in detail in this review (Table 1).

## 2 Non-Viral Nanocarriers for Nucleic Acid Therapeutics

To be effective, DNA needs to be delivered to the cell nuclei, whereas cytosolic release is required for RNA therapeutics. Both DNA and mRNA are used to induce expression of a gene of interest, while siRNA and miRNA can inhibit a specific gene expression *via* RNA interference (RNAi) [39]. To realize the tremendous potential of nucleic acid therapeutics, various classes of nanocarriers are being developed to facilitate transport to the site of interest in the patient and intracellular delivery of the nucleic acid payload to targeted cell types. The most established classes of nanoparticle-based delivery systems are lipid-based, polymeric, and inorganic nanoparticles [40]. Lipid-based nanoparticles include two main subsets of structures, liposomes and lipid nanoparticles (LNPs), both formed by self-assembly. While liposomes have a lamellar vesicular structure, LNPs, being the mostly widely used for nucleic acid delivery, consist of a micellar structure within the particle core [41,42]. Polymeric nanocarriers can be synthesized from either natural or synthetic materials and can accordingly be engineered with various structures and characteristics [43]. They can be divided into subsets based on nanoparticle structures, such as polymersomes, micelles, and dendrimers. Polymersomes consist of amphiphilic block copolymers that form a vesicular structure similar to liposomes [44]. Polymeric micelles also use block copolymers, which self-assemble into nanospheres with separation between the hydrophilicity of an interior core and an exterior shell [45]. Dendrimers, by contrast, are hyperbranched polymers forming three-dimensional structures whose shape, size, and charge can be controlled [46,47]. Dendrimers for nucleic acid delivery commonly use cationic polymers, and the nucleic acid molecules are loaded in the interior of the nanoparticle based on electrostatic interactions. Cationic polymers can also be used to form polyplexes with nucleic acids, forming non-dendrimer nanoparticles. Nanostructured inorganic nanoparticles, such as gold, iron, and silica can be used as both drug delivery systems and contrast agents for diagnostic purposes.

## 3 Nanoparticle Stability for Systemic Administration

Delivery vehicles for nucleic acid therapeutics need to be engineered to overcome biological barriers to reach targeted site to accomplish effective therapeutic treatments. Systemic administration is the most common route for drug delivery systems, since local delivery commonly involves more invasive procedures and complex techniques. Thus, nanoparticle-based delivery systems being administrated systemically need to ensure good colloidal stability to protect the nucleic acid payload during circulation, since unmodified nucleic acid molecules are susceptible to nucleases and are rapidly degraded upon administration *via* the blood stream. Thus, chemical modifications or nanocarriers are needed to protect nucleic acid-based therapeutics from being degraded [48,49]. In addition, nanocarriers can be engineered to prolong the blood circulation time of the formulation, enhancing their ability to interact with BBB and subsequently facilitate crossing [50]. Key design parameters

of nanoparticles for systemic delivery of nucleic acids to the brain include size and surface charge. Nanoparticles having a diameter smaller than 10 nm are generally rapidly cleared from circulation by the kidneys, whereas nanoparticles larger than 200 nm may activate the complement system and become removed from the blood stream [40,51]. Additionally, systemically administrated nanoparticles with a positive surface charge may aggregate or the presence of anionic serum protein can cause competitive binding, which in turn leads to nucleic acid dissociation from the formulation. The most widely established design principles for improving nanoparticle stability include crosslinking and polyethylene glycol (PEG) functionalization [52-54].

In one example of a crosslinking approach, it was recently demonstrated that photo-crosslinking could improve the nanoparticle stability of bioreducible poly(beta-amino ester) (PBAE) nanoparticles for systemic siRNA delivery [55]. The engineered crosslinked bioreducible nanoparticles (XbNPs) facilitated efficient siRNA-mediated knockdown under high serum conditions in both patient-derived and murine GBM cells.

The incorporation of PEG provides the nanoparticles with stealth functionality due to increased hydrophilicity and shielded surface charge, which prolong circulation time [56] by decreasing non-specific interactions between the nanoparticles and proteins and cells found in the blood. Because of this, however, PEG shielding can also lead to decreased interactions with the target cell type and, thus, lower cellular uptake [57]; an important consideration when using this strategy therefore is the optimization of the PEG length and grafting density to balance the stability and transfection efficacy. Targeting groups can additionally be conjugated directly to the PEG for synergistic effects, where the PEG linker creates an inner shell for improved stability, and the ligand, an outer shell for targeting. This approach of combining PEGylation with ligands has been used for BBB targeting; including with ligands Angiopep-2 (Ang), transferrin, and chlorotoxin (CTX) [58-61]. As an alternative to PEGylation, zwitterionic materials can be incorporated to reduce non-specific protein adsorption by reducing surface charge [62]. They can also be functionalized with targeting ligands of the endothelial cells of brain capillaries to both ensure good colloidal stability in circulation and facilitate BBB crossing. The approach of using Ang conjugated to the zwitterionic lipid distearoyl phosphoethanol-aminepolycarboxybetaine (DSPE-PCB) demonstrated synergistic effects by providing efficient systemic delivery of siRNA to brain tumors in an orthotopic mouse model [63].

In addition, surface modifications can also be used to avoid recognition of the mononuclear phagocyte system (MPS) and renal excretion of nanoparticles to prolong their circulation time. This can be achieved either by incorporating CD47 groups as a self-marker to block phagocytosis by macrophages [64] or by mimicking or incorporating cell-membrane coatings of red blood cells [65,66]. Liu *et al.* demonstrated the use of red blood cell membrane (RBCm)-coated nanocomplexes for improved siRNA delivery to the brain [67]. These RBCm coated nanocomplexes demonstrated an impressive blood-circulation time with an elimination half-life ($t_{1/2}$) of about 1.5 h, whereas naked siRNA was rapidly eliminated with $t_{1/2}$ = 5 min.

## 4 Blood-Brain Barrier Crossing

A major challenge in the development of therapeutic treatments for CNS disorders, including brain tumors, is to achieve sufficient transport across the BBB. Its unique barrier properties ensure homeostasis of the brain by regulating entry of substances essential for brain function and preventing toxins from reaching the brain [50,68]. The main challenge preventing potential drug candidates from extravasating into the brain is the presence of tight junction (TJ) proteins between adjacent endothelial cells of the BBB, which limits passive diffusion of substances from the blood to the brain [69]. The integrity of the BBB endothelium with its TJs restricts the entry of almost all macromolecules and over 98% of small molecule drug candidates [69-71]. Only lipid-soluble small molecules with a molecular weight <400 Da are able to cross, and naked nucleic acid-based therapeutics are thereby incapable of reaching the brain unaided [70]. Thus, drug delivery systems that can enable transport, such as active transcytosis across the BBB, are needed to enable unmet therapeutic needs for the treatment of brain tumors using nucleic acid-based therapeutics. To overcome the limitations of BBB crossing, nanoparticle-based drug delivery formulations can be used. Nanoscale delivery systems can be engineered to enable receptor-mediated transcytosis (RMT), carrier-mediated transcytosis (CMT), or adsorptive-mediated transcytosis (AMT) [72,73]. The most widely used approaches to enable BBB crossing by nanoparticles delivering nucleic acids are RMT and AMT. AMT can be achieved based on electrostatic interactions between the negatively charged BBB endothelium and positively charged nanoparticles. Additionally, nanoscale delivery carriers can also be functionalized with ligands to target receptors on the BBB endothelial cells for RMT [74-76]. In addition to targeting of the BBB endothelium to enable delivery to the brain, dual targeting of receptors of both the BBB endothelium and brain cancer cells can also be used to further restrict cellular targeting.

### 4.1 Ligands for Receptor-Mediated Transcytosis and Accumulation in the Tumor

The BBB endothelium expresses receptors to regulate transport of nutrients that are essential for brain functions. Some of these receptors and others are also overexpressed on glioma cells, and binding to glioma cells or other cells of interest in the brain can also help to enable BBB crossing. Thus, nanoparticle formulations can be functionalized with ligands to target these receptors and facilitate RMT across the BBB.

Ang is a peptide used to target low-density lipoprotein receptor-related protein (LRP), which is overexpressed by the BBB endothelium as well as by GBM cells; thus, providing dual-targeting functionality for both tissue-mediated delivery to the brain and also cellular targeting of glioblastoma cells. Qiao *et al.* used the reactive oxygen species (ROS)-responsive polymer poly[(2-acryloyl)ethyl(p-boronic acid benzyl)diethylammonium bromide] (BAP) as a nanocarrier for the co-delivery of siRNA targeting tumor growth factor $\beta$ (siTGF-$\beta$) and temozolomide (TMZ) [63]. Ang was conjugated to the maleimide groups of a zwitterionic lipid incorporated in the formulation. The authors first demonstrated *in vitro* that their Ang-functionalized nanoparticles (ALBTA) facilitated increased uptake in GL261 cells after crossing a cell monolayer mimicking the BBB endothelium. In a subsequent *in vivo* study using an orthotopic glioma mice model, their ALBTA formulations

enhanced the accumulation in the brain tumors following intravenous (IV) injections [63]. This selective delivery to the brain tumors extended the survival time when used for co-delivery of siTGF-$\beta$ and TMZ. Zheng *et al.* also functionalized their polymeric nanoparticles with Ang for dual targeting to enable active transcytosis through the BBB and cell-specific delivery of siRNA to brain cancer cells [60]. In their formulation termed Ang-3I-NM@siRNA, Ang was conjugated to poly(ethylene glycol) (PEG), where the block-copolymer used as the nanocarrier contained PEG and guanidinium (Gu). They first assessed the receptor-mediated delivery by Ang to glioma cell line U87MG *in vitro*, showing that their nanoparticle decorated with Ang promoted cellular uptake and subsequent silencing of the reporter gene luciferase. The ability of Ang-3I-NM@siRNA to facilitate crossing of BBB was demonstrated in an *in vitro* assay mimicking the BBB endothelium, in which nanoparticles decorated with Ang promoted BBB permeation [60]. This was further demonstrated *in vivo* using an orthotopic mouse glioma model in which IV-administered Ang-3I-NM@siRNA accumulated in the brain and specifically at the tumor site. This improved delivery to the tumor site increased the survival time in the mouse model when the nanoparticles co-delivered siRNA targeting polo-like kinase 1 (PLK-1), overexpressed in GBM, especially highly proliferative subtypes [77], and VEGF receptor 2 (VEGFR2), overexpressed in surrounding blood vessels. In a recent study, Zou *et al.* described a single siRNA complexed electrostatically with acrylate guanidine, then crosslinked with the disulfide-containing N,N'-bisacryloyl cystamine to form a nanocapsule [78]. These nanocapsules were then functionalized with Ang, and the authors termed the formed particles Ang-$NC_{ss}$(siRNA) being approximately 25 nm (Figure 2). Approximately 7% of the injected dose per gram tissue (%ID/g) following systemic administration of Ang-$NC_{ss}$(siRNA) was detected within the tumor volume. When they delivered siRNA targeting PLK-1, this delivery system led to PLK-1 knockdown and improved survival in mice bearing patient-derived xenografts [78]. In another related study, the authors designed nanoparticles to achieve a synergistic effect from Ang-mediated targeting and incorporation of a red blood cell membrane (RBCm) for prolonged circulation time [67]. The extended circulation time and Ang targeting led to tumor accumulation of their nanocomplex design and tumor inhibition when delivering therapeutic siRNA.

Another strategy targeting the LRP overexpressed by the BBB endothelium was reported by Jin *et al.*, who designed a solid lipid nanoparticle (SLN) reconstituted from natural components of protein-free low-density lipoprotein [79]. They demonstrated that their SLN formulation carrying siRNA reached the brain following IV injection in an orthotopic U87-MG mouse model. They further demonstrated that systemically administrated SLNs delivering therapeutic siRNA targeting c-Met reduced cell proliferation in the tumor and resulted in decreased tumor growth [79].

The transferrin receptor (TfR) of the BBB endothelium is another interesting target to direct the delivery system to the brain through RMT. Cai *et al.* reported that the T7 peptide can be used to specifically target TfR to achieve BBB crossing [61]. Additionally, the T7 peptide was incorporated using an acid-cleavable PEG, and following endocytosis, this acid-responsive linker detached from the nanoparticles separating them from the transferrin receptor to ensure brain entry. This nanoparticle design based on dendrigraft poly-L-lysine

(DGL) demonstrated synergistic effects whereby the combination of T7 peptide with the acid-responsive linker facilitated the highest siRNA delivery to the brain.

Transferrin functionalization can also be used for targeting many solid tumors, including brain tumors. The tumor targeting of transferrin-functionalized nanoparticles for siRNA delivery following systemic administration was first demonstrated in a clinical trial with melanoma patients [80]. The authors reported that cyclodextrin-based polymeric nanoparticles decorated with transferrin facilitated siRNA-mediated silencing in melanoma tumors. The dual-targeting functionality of transferrin for BBB crossing and targeted delivery to glioblastoma cells was demonstrated by Lam *et al.* in their nanoparticle design for co-delivery of TMZ and the bromodomain inhibitor JQ1 [59]. The authors reported that the transferrin-functionalized PEGylated nanoparticles (Tf-NP) enhanced the NP uptake in the brains of mice following IV administration. The ability of Tf-NPs to enable transcytosis across the BBB led to reduced tumor size and prolonged survival in an orthotopic glioma mouse model when co-delivering TMZ and JQ1. As another method of targeting TfR-expressing glioma cells, rather than using transferrin itself as the targeting ligand, Kim *et al.* used a single-chain variable fragment (scFv) specific for TfR to coat their cationic liposomes containing DNA encoding p53. Using fluorescently labeled oligonucleotides, they demonstrated transfection of glioma stem cells *in vivo*, and combination of this gene delivery system with the administration of the standard chemotherapy TMZ led to enhanced survival in a TMZ-resistant model [81].

Another targeting ligand with anti-glioma potential is CTX, a 36 amino-acid peptide [58,82] derived from scorpion venom that binds to glioma cells. Although the exact receptor(s) for CTX are unclear, its binding to glioma cells has been found to correlate positively with disease severity [83]. Its reported binding partners include voltage-gated calcium channels, MMP-2, and Annexin 2 [84,85], and CTX binding to tumor cells has several effects that may be therapeutically beneficial, such as reduced angiogenesis [83]. Conjugation of CTX to a cationic poly(amidoamine) (PAMAM) dendrimer *via* a PEG linker led to high uptake of DNA-containing nanoparticles in C6 rat glioma cells but not in a control cell line (293 cells). The authors also demonstrated that the CTX linkage caused greater *in vivo* transfection of a tumor with DNA encoding tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), leading to longer median survival compared to animals treated with TMZ [86]. When used to functionalize stable nucleic acid lipid particles (SNALPs) for intracranial miRNA delivery, CTX again enhanced uptake into rodent glioma cell lines but not healthy tissue *in vitro* and *in vivo*, and transfection was efficient enough to cause decreased proliferation [87]. Stephen *et al.* used this targeting strategy with a different type of nanoparticle, containing an iron oxide core coated with a chitosan-PEG-polyethylenimine (PEI) co-polymer with CTX ligand, and this functionalization enabled siRNA delivery to the brain after systemic injection in a mouse (C57BL/6) model [82]. The CTX-conjugated nanoparticles mainly accumulated in the tumor region of the brain, showing that this ligand could facilitate crossing of the BBB as well as tumor-cell targeting. In their therapeutic model, the authors explored the use of siRNA targeting the protein $O^6$-methylguanine-DNA methyltransferase (MGMT) in combination with TMZ, as MGMT activity can confer resistance to TMZ therapy [88]. Synergistic effects were observed with the combinational delivery of siMGMT and TMZ, leading to 5-fold smaller tumor volumes and prolonged survival of 18.3 days

compared to TMZ alone, which only extended lifespan by 2.3 days. They also demonstrated the use of this nanoparticle design conjugated with a CTX moiety for DNA delivery, which significantly improved DNA transfection of a tumor after systemic injection [89]. Interestingly, the authors noted that, while gene expression increased in the tumor when CTX was conjugated to the nanoparticles, total particle accumulation did not, indicating that the CTX-derived specificity in this case was due to an increase in uptake efficiency, not tissue accumulation [89].

Preferential accumulation in the brain and cell specificity has also been reported for liposomal nanoparticles functionalized with RGDK-lipopeptide [90]. This ligand targets the $\alpha 5\beta 1$ integrin receptors expressed on both glioma cells and tumor vasculature. In an orthotopic glioblastoma *in vivo* model, the authors demonstrated that approximately 13% of the injected dose per organ weight accumulated in the brain when nanoparticles were functionalized with RGDK ligands [90]. The RGDK-liposomes used for co-delivery of siRNA targeting STAT3 and WP1066 (a small-molecule STAT3 inhibitor) significantly extended the survival time in mice bearing GL261 tumors.

RMT across the BBB can also be achieved by targeting glucose-transporter 1 (GLUT1), which is expressed by the brain capillary endothelial cells. GLUT1 undergoes translocation from the apical to the basal side of the BBB endothelium in response to blood glucose level [91]. Accordingly, optimizing the density of glucose ligands on a nanoparticle formulation can give rise to effective BBB transcytosis [92].

Another molecule that can be used to increase accumulation in brain tumors after systemic injection is neuropilin-1, which is overexpressed on a wide variety of cells and is upregulated in some malignancies, including in the U87 human GBM cell line. Wang *et al.* screened C-end rule (CendR) peptides with the C-terminal motif R/KXXR/K, where "R/K" is arginine or lysine and "X" is any other amino acid, for neuropilin-1 binding [93]. They found that the peptide RGERPPR, when conjugated to PEG-PEI and used to condense DNA into nanoparticle, enhanced U87 transfection *in vitro* [93]. A retro-inverso CendR peptide, which contains D-amino acids in reverse order from the original L-amino acid sequence and is resistant to proteolysis, also showed neuropilin-1 binding and enhanced U87 transfection with DNA compared with unconjugated PEG-PEI nanoparticles (Figure 3) [94]. When used to deliver TRAIL DNA in a U87-bearing mouse model of glioma, the authors observed approximately 20% increase in survival time [94].

Although many therapies are meant to affect the malignant tumor cells themselves, other cell types, particularly microglia [95], can also be targeted. Microglia, which make up 5-20% of the glial cells in the brain [96], are a predominant immune cell type in the brain [97]. They take up high levels of extracellular vesicles (EVs) from glioma cells, which has been shown to be one method by which they are genetically reprogrammed by the tumor [98,99], and their prominent role in the tumor microenvironment makes them an attractive cell type for anti-glioma therapies. A virus-inspired nanogel, called Vir-Gel, was used to target delivery to microglia for genetic reprogramming [100]. The Vir-Gel was fabricated from poly(caprolactone) (PCL) grafted with DNA and cross-linked with miRNA containing complementary overhangs to form nanogels. These were then

coated with erythrocyte membranes and functionalized with M2pep, which targets M2-type macrophages and microglia, and the HA2 peptide, which promotes membrane fusion (Figure 4). The Vir-Gel was then used to deliver miR-155, which promotes a pro-inflammatory and anti-tumor M1 phenotype, and the authors showed both high accumulation of the particles in the brain after IV injection as well as decreased tumor growth. In particular, they noted an increase in microglia expressing high levels of CD86, an activation marker, and a decrease in microglia expressing high levels of CD206, an M2 phenotypic marker [100].

Finally, RMT can also be achieved without functionalization with ligands. Jensen *et al.* reported the use of 13-nm gold nanoparticles with siRNA covalently bound, enabling BBB crossing following systemic administration [101]. Their nanoparticle design, termed spherical nucleic acid (SNA), facilitated higher accumulation in brain tumors than in healthy brain tissue in a tumor-bearing mouse model, and the delivery siRNA targeting oncoprotein Bcl2-like protein 12 (Bcl2L12) resulted in slowed tumor progression. Choi *et al.* demonstrated that the mechanism by which this nanoparticle design mediates endocytosis and transcytosis is *via* the binding of scavenger receptors [102]. This design of SNA carrying siRNA targeting Bcl2L12 administered systemically was recently used in a first-in-human phase 0 clinical trial as treatment for patients with recurrent GBM [103]. The authors reported that the IV-administered SNA reached patient tumors and that the uptake of SNA in glioma cells correlated with reduced Bcl2L12 protein expression, showing its potential in the clinic for systemic treatment of GBM.

### 4.2 Physical and Chemical Nanoparticle Properties for Adsorptive-Mediated Transcytosis

Size and surface charge are key characteristics of nanoparticles for achieving active transcytosis across the BBB. Cationic nanoscale delivery systems can potentially facilitate AMT based on electrostatic interactions with the BBB endothelium. This mechanism mimics the natural transport of polycationic proteins, such as protamine, which binds to the BBB endothelial cell surface, resulting in cellular uptake and transport into the brain parenchyma [104,105]. Our group recently demonstrated that bioreducible PBAE nanocarriers enable systemic delivery of siRNA to brain tumors by adjusting the mass ratio of the cationic polymer to the anionic siRNA load, tuning both the size and surface charge of the nanoparticle formulations [106]. In an *in vitro* BBB model using derived human brain microvascular endothelial cells (dhBMECs) seeded as a monolayer in transwells, it was demonstrated that the engineered nanoparticles facilitated AMT across the endothelial monolayer. In an orthotopic brain tumor model, the bioreducible PBAE-based nanoparticles facilitated systemic siRNA delivery to the brain tumor, and the particles were found to be distributed through about 50% of the tumor volume [106]. In another study, the use of cationic albumin-conjugated pegylated nanoparticles (CBSA-NP) carrying DNA was demonstrated to facilitate delivery to the brain upon IV administration in mouse model and that the cationic albumin component promoted the accumulation of nanoparticles [107]. The authors mechanistically explored the BBB crossing of their nanoparticle design and showed that the CBSA-NPs colocalized with the negatively charged glycoproteins of the brain capillary endothelial cells and were subsequently transported in endolysosomal compartments across the BBB endothelium, supporting the mechanism of AMT. Interestingly, the negatively charged glycoproteins were upregulated both in the tumor

vasculature and on the tumor cells, which resulted in higher accumulation of CBSA-NPs in the brain tumors [107].

### 4.3 Intranasal Delivery

An alternative strategy is to bypass the BBB *via* intranasal administration of nanoparticles. In this approach, the nanoparticles are instead engineered to cross the olfactory epithelium *via* the olfactory or trigeminal nerve system to reach the brain for treatment of brain tumors and other CNS disorders. The direct targeting offered by the intranasal route can potentially enhance the efficacy of neurotherapeutics, including nucleic acid therapeutics. This approach has shown promise for siRNA delivery as a treatment for brain tumors [108-110]. An alternative strategy was reported by Mangraviti *et al.*, in which the authors transfected human adipose-derived mesenchymal stem cells (hAMSCs) *ex vivo* using polymeric nanoparticles containing bone morphogenetic protein 4 (BMP4) plasmid DNA [111]. They demonstrated *in vivo* that rats bearing brain tumors and treated with engineered hAMSCs *via* intranasal administration had extended survival time. Despite the promise of intranasal administration for treatment of brain tumors, there have been a rather limited number of studies that have explored its use for nanoparticle formulations encapsulating nucleic acid-based therapeutics. For an interested reader, we recommend other reviews that have thoroughly discussed the potential of using the intranasal route to target the brain for various drug delivery systems and biologics [112,113].

## 5 Nanoparticle Diffusivity in Brain and Tumor Tissue

Following extravasation into the brain and the tumor site, nanoparticles must then diffuse through the brain tumor region or the brain tissue to reach the targeted cells. This is also critical when nanoparticles are delivered *via* local administration, which can be used to increase particle concentrations at the target site and reduce the total dose needed of the therapeutic [114]. Though this route has limited clinical application since it in many cases would involve an invasive procedure, as described above, local administration can be coupled with initial surgical resection of the tumor, part of the standard of care for most brain tumors. Although controlled-release depots can be and are regularly implanted during surgery in the clinic, [115,116], these systems also demonstrate the transport hurdles for drugs relying primarily on diffusion through the brain. A modeling study showed that high concentrations of carmustine, a small-molecule drug, could be measured immediately surrounding a Gliadel®-based implant but that concentrations dropped steeply with increasing distance, with the steepness of the drop-off depending on properties of the drug [117]. While this can be advantageous for reducing neurotoxicity on healthy brain tissue, it also limits the probability of killing malignant cells that have migrated or invaded more distant regions of the brain. Notably, given the larger size of nanoparticles compared to small molecules, diffusivity is expected to be even lower for nanoparticles than for conventional chemotherapeutics if unaided.

Key characteristics of the nanoparticle formulation to consider for increasing diffusivity in the brain are particle size, surface charge, and shape. Our lab demonstrated the use of bioreducible PBAE nanoparticles carrying miRNA for intratumoral delivery in an orthotopic

human GBM xenograft model [118]. The presence of bioreducible bonds in the polymer structure allowed the use of a higher weight ratio (w/w) of polymer to miRNA in the nanoparticles without concerns about toxicity. The higher w/w ratio resulted in nanoparticles that complexed miRNA more strongly, resulting in a smaller nanoparticle size than that of their non-bioreducible counterparts. The engineered bioreducible nanoparticles delivering miRNA were found to be distributed through approximately 60% of brain tumor volume (Figure 5) [118]. In other studies, PBAEs have been used to form nanoparticles with DNA delivered *via* CED, facilitating diffusion through tumor volumes in glioma-bearing rats [119]. Following a single CED infusion, the PBAE-based nanoparticles spread throughout the tumor region, reaching the margins, and transfected the tumor mass specifically due the pressure gradient created by CED, which enhances diffusion throughout the tumor mass. Related polymeric nanoparticles can also be engineered for CED and gene editing in mice bearing murine glioma tumors [120]. Carboxylated branched PBAE nanoparticles were used to encapsulate CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 ribonucleoproteins (RNPs), and gene editing was achieved in the orthotopic glioma tumors and detected several millimeters away from the primary injection site. In another study, Yu *et al.* showed the therapeutic potential of CED by administrating lipopolymeric nanoparticles carrying siRNAs, which significantly extended survival in a xenograft GBM mouse model [121].

Another promising design principle is to use PEGylated nanoparticles to shield the surface charge of the nanoparticles for improved penetration of the brain tissue. Kim *et al.* demonstrated that PEGylation of PBAE-based nanoparticles improved their diffusion in the brain, reaching up to 2 mm from the injection site [122]. In addition, the PEG-PBAE nanoparticles penetrated 28.5% of the tumor volume whereas the non-PEGylated nanoparticles only covered 14.3% of the tumor volume, and the PEG-PBAE nanoparticles extended survival following CED in an orthotopic human glioblastoma model in mice.

## 6 Cell Targeting and Intracellular Trafficking

Once the drug or delivery vehicle has reached the brain, whether by crossing the BBB after systemic administration or by local placement near the tumor site, it must target the cell type of interest for successful and specific transfection. Many targeting methods rely on ligands that bind to the cell type(s) of interest. However, some researchers have also reported higher levels of transfection of cancer cells or tumor tissue compared to their healthy counterparts without explicitly designing targeting functionality. For example, using a nanoparticle library approach and high-throughput screening methods, our group has identified cationic biodegradable PBAEs that are highly effective for DNA delivery to patient-derived human GBM cells *in vitro* [123]. It was demonstrated empirically that these materials chemically optimized for delivery to glioma cells *via* screening were approximately 5-fold more effective at transfection of brain tumor initiating cells than non-cancerous neural progenitor cells (NPC) *in vitro* and demonstrated statistically significant specificity for malignant over healthy brain tissue *in vivo* [119,124]. Similarly, PBAEs developed for siRNA-mediated gene knockdown in human GBM cells were also found to be less effective at transfecting NPCs [125]. Yu *et al.* used a lipopolymeric nanoparticle called 7C1, a biomaterial discovered by screening over 500 lipopolymeric compounds

for transfection, and reported enhanced delivery with 7C1 *in vivo* to brain tumor cells compared to non-cancerous cells following CED [121]. As was also the case with PBAEs, the authors acknowledged that the ligands or biomaterial properties that promoted brain cancer specificity using the lipopolymeric nanoparticles are still unknown, though the selectivity was demonstrated empirically. While high-throughput methods can be used to screen compounds in this way, the lack of mechanistic understanding of this specificity complicates the design of such nanoparticles. Cell-penetrating peptides (CPPs), such as the HIV Tat peptide, can facilitate greater uptake of nanoparticles by cells in general [126]; for instance, Wang *et al.* used CPPs to complex DNA, leading to greater *in vitro* transfection. However, this method may not provide specificity exclusively to brain cancer cells.

### 6.1 Cell-Specific Ligands

A common way to achieve cell-specific transfection is to capitalize on differences in surface protein expression on tumor cells or microglia by designing delivery vehicles that bear particular ligands. The use of targeting ligands to promote accumulation in the tumor after crossing the BBB is detailed above. However, this type of strategy can also be used to improve cell targeting and intracellular delivery after local injection.

The folate receptor (FR) is overexpressed on many glioma cells and other malignancies [127]. A folic acid (FA)-PEG conjugate was grafted to hyperbranched, cationic PEI and used to condense plasmid DNA into nanoparticles. The presence of FA on the particles improved transfection of C6 rat glioma cells, which overexpress FR, but not of HepG2 cells, which do not [128]. A similar type of particle, composed of FA-PEG conjugated to linear PEI, was used to deliver siRNA against BCL-2 (B-cell lymphoma-2), an anti-apoptotic gene. Following preferential accumulation of the siRNA-loaded nanoparticles at the glioma site, the authors reported that knockdown of BCL-2 led to decreased resistance to doxorubicin [129].

CD44 is overexpressed in glioma cells compared with healthy astrocytes and is important for glioma migration and invasion [130]. Hayward *et al.* showed that liposomes functionalized with hyaluronic acid (HA) were preferentially taken up by glioma cell lines U87, U251, and A172 rather than by healthy astrocytes [131]. A similar targeting concept was also used by Cohen *et al.*, who grafted HA to lipid nanoparticles (LNPs) for glioma therapy [132]. These LNPs were used to deliver siRNA against PLK1, which plays a role in the malignant transformation of glioma cells, and local CED of the HA-functionalized LNPs significantly extended survival in the U87-bearing mouse model [132].

### 6.2 Intracellular Delivery

Once nanoparticles are taken up by cells, they must still overcome certain delivery hurdles in order for the particles and their cargo to localize to the correct intracellular sites. A major barrier to efficient non-viral gene delivery is endosomal escape [133-135]. Most nanoparticles are taken up into endosomes, where they may be degraded by the acidic compartment. The Vir-Gel delivery system mentioned previously used the HA2 peptide to promote fusion with the endosomal membrane in order to escape the endosome [100]. Aside from membrane fusion, another common strategy is the use of reversibly protonated

polymers, lipids, or dendrimers, which can buffer the endosomal pH. According to the proton sponge hypothesis, by accepting the protons that are pumped into endosomes, these types of buffering materials drive an influx of other ions and water *via* electrostatic and osmotic gradients, finally resulting in rupture of the endosome and escape of the particle into the cytosol [136]. For instance, Routkevitch *et al*. showed that an acidic microenvironment hinders endosomal escape capacity but that this effect can be mitigated by polymeric transfection agents, such as PBAEs, that can act as buffers in the physiological pH range [137]. Another study using PBAEs to deliver miRNA to cancer stem cells within a glioma used imaging to demonstrate that these reversibly cationic polymers promote highly efficient endosomal escape [118]. In this case, co-delivery of miR-148a and miR-296-5p using PBAEs significantly decreased tumor growth and increased survival in a patient-derived xenograft model of GBM in mice [118]. pH-sensitive lipid carriers can also take advantage of this mechanism, such as in the case of siRNA-loaded cationic lipid-based nanoparticle that was reported to be able to escape the endosome after particle uptake in U87 GBM cells [138]. Reversibly protonated dendrimers can cause the same effect via the proton sponge mechanism [139], and additional cationic moieties based on arginine or histidine can also be used to further improve endosomal escape [140].

Other researchers have used cationic polymers like PEI in combination with other materials, such as SNAs, as the reversible protonation may facilitate improved endosomal escape [141]. Interestingly, although this group has shown that SNAs on their own accumulate in endosomes, these hybrid nanoparticles do take advantage of other SNA properties, such as their ability to be taken up efficiently due to the organized nucleic acid structure on the particles, and this formulations also decreased the toxicity of PEI per mass of polymer, allowing the use of greater amounts of the material [142].

After escaping the endosome and entering the cytosol of cells, the nucleic acids must then be released from the delivery vehicle in order to take effect or else be further trafficked to other subcellular compartments. A cytosolic release strategy that has been explored by numerous researchers takes advantage of the relatively reducing environment of the cytosol, where the reducing agent glutathione (GSH) is present at concentrations roughly three orders of magnitude higher than in the extracellular environment [143]. PBAEs containing reducible disulfide linkages at the end-termini of the polymers were found to be particularly effective for *in vitro* siRNA delivery to patient-derived GBM cells [144]. Even more efficient were PBAEs with disulfide bridges all throughout the backbone of the polymers, and Kozielski *et al*. demonstrated that this not only improved siRNA transfection efficacy but also dramatically reduced toxicity compared to non-reducible counterparts, likely due to the rapid degradation of the polymer once inside the cell [145]. A similar polymer showed successful miRNA delivery not only *in vitro* but also *in vivo* in a GBM model, leading to improved survival [118]. Other researchers have also taken advantage of reducible disulfide bridges in their materials for nucleic release or rapid material degradation inside the cell, such as a nanoparticle based on branched PEI functionalized with a cyclic RGD-PEG moiety linked via disulfide bridges for plasmid DNA delivery to U87 cells *in vivo* [146]. Disulfide linkages were also used to load siRNA sequences onto quantum dots, leading to rapid siRNA release following internalization into U87 cells [147].

### 6.3 Cancer Cell-Specific Activity

Another strategy for targeting cells is to ensure that the cargo being delivered is only active in the desired cell type. DNA can be designed so that the gene of interest is under the control of a cell-specific promoter and will only be transcribed in certain types of cells. As an example, H19 and insulin-like growth factor 2 (IGF2) are both overexpressed in many cancer types, including glioma. By placing the diphtheria toxin A (DTA) gene under control of promoters active only cells in expressing H19 and IGF2, Amit *et al*. reported higher expression and specific cytotoxicity in U87 glioma cells when the DNA was carried by the commercially available cationic polymer JetPEI, resulting in better anti-tumor activity in a subcutaneous U87 model in mice [148].

Aside from transcriptional targeting, the gene being delivered can be selected so that the expressed protein is only effective in select cells. TRAIL, for instance, mentioned previously, causes apoptosis only in cells that overexpress the death receptors DR4 and DR5. This includes many cancer cells, though, as a caveat, these death receptors have also been found to be expressed in some normal tissues [149], while some cancer cells are resistant to TRAIL due to downregulation of one or both death receptors and/or upregulation of decoy receptors that bind TRAIL but do not lead to apoptosis [150]. However, TRAIL delivery remains a frequently investigated strategy for glioma therapy and may be combined with other targeting mechanisms, such as CTX [86] or RGD [151] functionalization of nanoparticles to kill tumor cells *in vitro* or *in vivo*. Similarly, apoptin, derived from the chicken anemia virus, causes apoptosis specifically in cancer cells. Though its exact mechanism of action is still subject to debate, it is believed to be phosphorylated by a kinase that is present only in cancer cells, leading to aggregation and, ultimately, apoptosis [152]. DNA encoding apoptin has also been delivered to glioma cells using polylysine- or polyglutamate-modified PAMAM dendrimers [153,154] to cause cancer-specific cell killing.

Finally, overexpression of certain genes by brain cancer cells can also be targeted directly by delivery of siRNA to knock down expression of genes that contribute to malignancy, migration, or growth. More than half of primary human GBM tumors overexpress the epidermal growth factor receptor (EGFR), leading to excessive growth, and the oncogenic mutation EGFRvIII is expressed only in tumor cells [155]. EGFRvIII has therefore been targeted by multiple groups using siRNA. In one case, cyclodextrin-modified dendritic polyamines (DexAMs) were used to deliver siRNA against EGFRvIII and was combined with small molecule delivery to achieve a synergistic anti-tumor effect [156]. Jung *et al*. also delivered siRNA against EGFRvIII using multifunctional quantum dots (QDs) [147]. Their delivery platform incorporated multiple mechanisms of targeting, including an RGD signal to improve uptake by U87 cells, an HIV-Tat peptide to improve membrane fusion for uptake and endosomal escape, and reducible linkages for quick cytosolic release of siRNA [147] (Figure 6).

### 6.4 Cell-Mediated Targeting of Brain Cancer Cells

Other cell types can be transfected non-virally with nanoparticles, either *ex vivo* or *in vivo*, and then used to target brain cancer by a variety of mechanisms. These include T cells and

dendritic cells (DCs) for glioma-specific immunotherapy as well as stem cells that home toward cancer cells.

**6.4.1 CAR T Cells—**CAR T cells have been explored as a method of targeting GBM and other brain cancers. In order to generate these cancer-specific T cells, genetic engineering of T cells is required and has been explored using multiple different types of viral or non-viral nanoparticle systems. Most studies of CAR T-cell therapy for treatment of glioma thus far have used viral vectors for gene delivery, and autologous T cells have been transduced *ex vivo* and then re-administered; however, such studies illustrate the high potential impact of CAR T-cell therapy, and a growing number of groups have reported the use of non-viral nanoparticles for CAR T-cell manufacturing. Given that EGFRvIII is expressed in approximately 30% of GBM tumors, viral vectors have been used to transduce T cells to target EGFRvIII in human GBM-bearing immunocompromised mice [157] and even in patients [29]. However, while these clinical studies showed that the cells did traffic to the brain, efficacy was limited by changes in the immune microenvironment in response to CAR T cells therapy. T cells engineered in this way can also be designed to have other functionalities, such as the expression of a TGF-β trap protein, which leads to greater M1 polarization among tumor-infiltrating microglia, in addition to EGFRvIII targeting *via* the CAR [158]. Trivalent CAR T cells have also been designed to be able to target multiple glioma-related antigens, Her2, EphA2, and IL13Rα2, at the same time [159], thus helping to address the high variability in tumor antigen expression among patients. In another case, T cells were engineered to express a CTX peptide and were thus targeted *via* CTX binding rather than through TCR interactions, because CTX has been shown to bind to a greater variety of GBM subtypes than CARs specific for particular common glioma-associated antigens [160].

Because of the risks and high burden of manufacturing of lenti- and retroviral vectors for gene delivery, other research groups have explored the engineering of CAR T cells *via* electroporation for gene delivery. These often use transposon systems that can lead to genomic integration of the genes delivered. The *Sleeping Beauty* (SB) transposon was shown to have nearly random integration, in contrast to lenti- and retroviral vectors, which have a high rate of integration into cancer-related genes [161]. Using this system, Caruso *et al.* introduced genes for a CAR specific for EGFRvIII as well as the SB transposase into T cells via electroporation, resulting in CAR T cells with anti-tumor activity in mice with shorter manufacturing time than that needed for traditional viral methods [162]. The piggyBac transposon system, which has been found to have greater transposition activity than the SB system [163], has also been used to *via* nucleofection to generate T cells specific for CD133, expressed particularly on stem-like populations within gliomas [164].

Numerous nanoparticle-based technologies are currently in development for non-viral transfection of T cells, which could eventually be used for generation of CAR T cells for brain cancer therapy. A range of cationic polymers with different architectures was shown to have up to 50% DNA transfer efficacy to immortalized Jurkat cells *in vitro*, including poly(2-hydroxyethyl methacrylate) grafted with 2-(dimethylamino)ethyl methacrylate (DMAEMA), branched PEI, linear poly(DMAEMA), and comb- and sunflower-shaped pHEMA-g-DMAEMA [165]. In an interesting study, a PBAE was used for *in situ* generation

of CAR T cells, bypassing not only viral manufacturing but also *ex vivo* culture [166]. Here, the authors used the positive surface charge of the PBAE/DNA based nanoparticles to electrostatically coat them with an antibody against CD3 for T-cell targeting, as well as a nuclear localization signal (NLS) for better trafficking to the nucleus [166]. A similar method was used for mRNA rather than DNA delivery using a PBAE, resulting in transient CAR T cells [167]. Other groups have also engineered transient CAR T cells with mRNA by using lipid nanoparticles with ionizable lipids as the delivery vehicle and showed effective transfection of Jurkat cells [168].

**6.4.2 Dendritic Cells—**Dendritic cell (DC)-based vaccines are another attractive option for eliciting a targeted immune response against particular glioma-associated antigens. Once transfected to express the antigen of interest, DCs can be administered to the patient and present the antigen to T cells, leading to glioma antigen-reactive T cells. Much of the work on DC-based genetic vaccines for brain cancer have used electroporation or nucleofection for gene transfer, but non-viral nanoparticle-based methods are also under development. In one study, nucleofection was used to deliver mRNA encoding CD133 to murine bone marrow-derived DCs (BMDCs), which caused a cytotoxic immune response to CD133-expressing glioma stem cells upon administration to mice [169]. Electroporation has also been used to load glioma mRNA into patient-derived DCs in clinical trials and then re-administered [170,171], with some increase in survival seen and no reported adverse side effects.

While the use of non-viral nanoparticles to transfect DCs for brain cancer therapy is still in its infancy, a variety of biomaterials that have been developed for DC transfection could be applied to brain cancer as well. Saka *et al.* used a commercial lipid-based transection reagent, TransMessenger® Transfection Agent (Qiagen), to transfect primary murine BMDCs with mRNA encoding IL13Rα2 *ex vivo*. When the transfected DCs were injected intraperitoneally into mice with established intracranial tumors, the authors saw significantly longer survival as well as an increase in $CD8^+$ and $CD4^+$ T cells in the tumors [172], suggesting an adaptive anti-tumor immune response. Cationic liposomes have also been used to transfect up to 57% of BMDCs from mice with DNA or RNA and were found to be more effective than another lipid-based commercial agent, Lipofectamine™ 2000 (Life Technologies), and these *ex vivo* genetically engineered DCs were used to treat a mouse melanoma model [173]. Solid lipid nanoparticles (SLNs) have also been made by emulsion, then complexed with DNA for transfection of the DC2.4 murine cell line *in vitro* [174]. In addition to lipid-based materials, nanoparticles with DC-targeting moieties have been used to improve transfection. Because DCs express high levels of mannose receptor, mannose has been explored as a targeting ligand to improve uptake. It was found that the mannose-mimicking shikimoyl ligand was even more effective than a mannosyl analog for DC transfection [175], and it was therefore used to surface-functionalize gold nanoparticles (AuNPs), leading to up to 20% transfection of DCs for vaccination [176].

**6.4.3 Stem Cells—**Aside from immune cells, several types of stem cells have also been used as delivery vehicles to target brain cancer. It was discovered that neural stem cells (NSCs) exhibit intrinsic tropism toward gliomas [177]. Bone marrow-derived mesenchymal

stem cells (MSCs) also displayed similar patterns of tropism and could be collected from adult patients with fewer ethical concerns [178]; further, adipose-derived stem cells (ADSCs) were shown to be similar to bone marrow-derived MSCs in their safety and tumor-homing properties, and they could be collected from patients in high quantities using much easier and less invasive procedures [179]. Interestingly, MSCs and ADSCs often have an immunosuppressive phenotype, particularly in the tumor microenvironment, and it is unclear if their homing toward tumors may lead to potential pro-tumorigenic functions. However, their low immunogenicity permits the potential for the use of allogenic as well as autologous cells, though some immune response to these cells is still possible [180]. Transfected NSCs, MSCs, and ADSCs can home toward gliomas, even following tracks of migrating tumor cells, and deliver an exogenous protein locally to the tumor site.

Viral transduction methods have been used to transduce NSCs and NSC-like cells derived from bone marrow to express immunoactive cytokines like IL-12 and IL-23, a member of the IL-12 superfamily, leading to improved survival in mouse models [181,182]. Multiple groups have investigated the use of ADSCs as a means of delivering the TRAIL protein to brain cancers, including GBM and sarcoma, by virally transducing the stem cells with the TRAIL gene [183,184].

More recently, non-viral nanoparticles have also been used for *ex vivo* transfection of stem cells for GBM targeting. Jiang *et al.* used cationic PBAE to transfect ADSCs with TRAIL-encoding plasmid DNA and observed tropism toward tumors in a murine GBM model as well as tracking of tumor microsatellites [185]. By combining the targeting capacities of ADSC homing and TRAIL cancer-specificity, the authors were able to demonstrate apoptosis in the main tumor and microsatellites but not in healthy normal brain parenchyma, and this led to significantly longer survival in mice bearing a patient-derived GBM xenograft (Figure 7) [185]. In another study, a different PBAE was used to transfect ADSCs with bone morphogenic protein 4 (BMP-4), which causes terminal differentiation of brain cancer stem cells and therefore prevents recurrence of the tumor. The transfected ADSCs were then administered intranasally or intravenously and were found to accumulate at the tumor site and significantly increase survival in F98-bearing rats [186]. ADSCs can also be transfected to express a suicide gene, such as HSV-tk. Malik *et al.* used PLL-PEI as a cationic polymer to co-deliver genes encoding HSVtk and TRAIL in plasmid DNA to rat MSCs, combining the TRAIL and suicide gene strategies. The transfected stem cells were then administered intratumorally into C6 glioma-bearing rats, leading to significantly longer survival in animals treated with the transfected cells along with the prodrug ganciclovir [187].

## 7 Conclusions and Future Perspectives

Cancers of the CNS are among the most common and comprise many different subtypes, each of which has different properties that can affect the desired characteristics of a therapeutic. Among high-grade brain cancers, adult GBM and other gliomas have been most extensively studied in pre-clinical work, due in part to the availability of animal models. Even with the gold standard of care, the prognosis for stage IV GBM remains poor and has improved only marginally over the past years. Gene therapy has risen to

prominence in recent years for its versatile range of effects; in particular, non-viral gene delivery vehicles, including lipid- and polymer-based nanoparticles, are being pursued for their design flexibility and advantageous safety profiles. Several types of therapeutic strategies can be affected by gene therapy, including immunotherapy *via* gene delivery of immunoactive cytokines or transfection of T cells and antigen-presenting cells; local suicide gene therapy; knockdown of overexpressed genes that lead to malignancy or growth; and targeting of cancer stem cells that are often resistant to traditional radio- and chemotherapy. Because surgery is usually a first treatment for patients with this disease, local delivery strategies, which allow the bypassing of the BBB, a major challenge for delivery to the brain, are clinically feasible. At the same time, multiple delivery strategies have been employed to improve the ability of gene delivery nanoparticles to cross the BBB and bind to cancer cells or other cells of interest in the tumor microenvironment. Non-cancer cells can also be transfected *ex vivo* and used as cellular therapies to treat brain cancer, with certain stem cells having tropisms for targeting GBM tumors. The vast chemical diversity afforded by synthetic non-viral nanoparticles allows these delivery vehicles to be tailored very precisely for particular cellular targets and therapeutic strategies.

Synthetic nanoparticles and genetic engineering hold vast potential for the treatment of brain cancer. The advancement of synthetic gene-delivery nanoparticles into the clinic in other areas, such as for genetic vaccines [188], further eases the translational pathway for anti-cancer genetic medicines and increases the acceptance of these types of technologies into the arsenal for brain cancer treatment. A major hurdle that must still be overcome in order for gene therapy to be more extensively used in the clinic is improved efficacy and safety of the nanoparticle carriers. While many gene delivery technologies have relied on either viruses or lipid nanocarriers, both of these carry the risk of unintended adverse side effects to the patient, some of which have been reflected in the clinic. On the other hand, biodegradable polymeric systems have safety advantages due to their degradability and are often more versatile in their chemistry to tune functionality, but may be less efficient. Recent research has uncovered polymeric nanoparticle structures modified for greater gene delivery efficacy, as well as methods of modifying lipid-based nanoparticles for improved safety. Moreover, there are multiple routes by which nucleic acid delivery can be employed for brain cancer treatment, including direct administration of nanoparticles to the tumor, systemic administration of nanoparticles to non-malignant cells that can affect tumor progression, and even *ex vivo* engineering of cells that can subsequently be administered to a brain cancer patient, each of which may require a delivery vehicle with different characteristics. A final potential challenge for the translation of gene-delivery nanoparticles is scale up and manufacturing of a robust and stable product, including long-term stability during storage and transport. With ongoing improvements in the understanding of nanoparticle design and engineering and in brain cancer biology, gene-delivery technologies are poised to make a large impact on brain cancer patients and their treatment.

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## Abbreviations

| | |
|---|---|
| **AMT** | adsorptive-mediated transcytosis |
| **Ang** | Angiopep-2 |
| **BBB** | blood-brain barrier |
| **CNS** | central nervous system |
| **CAR** | chimeric antigen receptor |
| **CTX** | chlorotoxin |
| **CED** | convection-enhanced delivery |
| **FR** | folate receptor |
| **GBM** | glioblastoma |
| **LNP** | lipid nanoparticle |
| **GLUT1** | glucose-transporter 1 |
| **GSH** | glutathione |
| **LRP** | lipoprotein receptor-related protein |
| **PBAE** | poly(beta-amino ester) |
| **PEG** | polyethylene glycol |
| **PEI** | polyethylenimine |
| **RMT** | receptor-mediated transcytosis |
| **SNA** | spherical nucleic acids |
| **TJ** | tight junction |
| **TMZ** | temozolomide |
| **TfR** | transferrin receptor |
| **TRAIL** | tumor necrosis factor-related apoptosis-inducing ligand |

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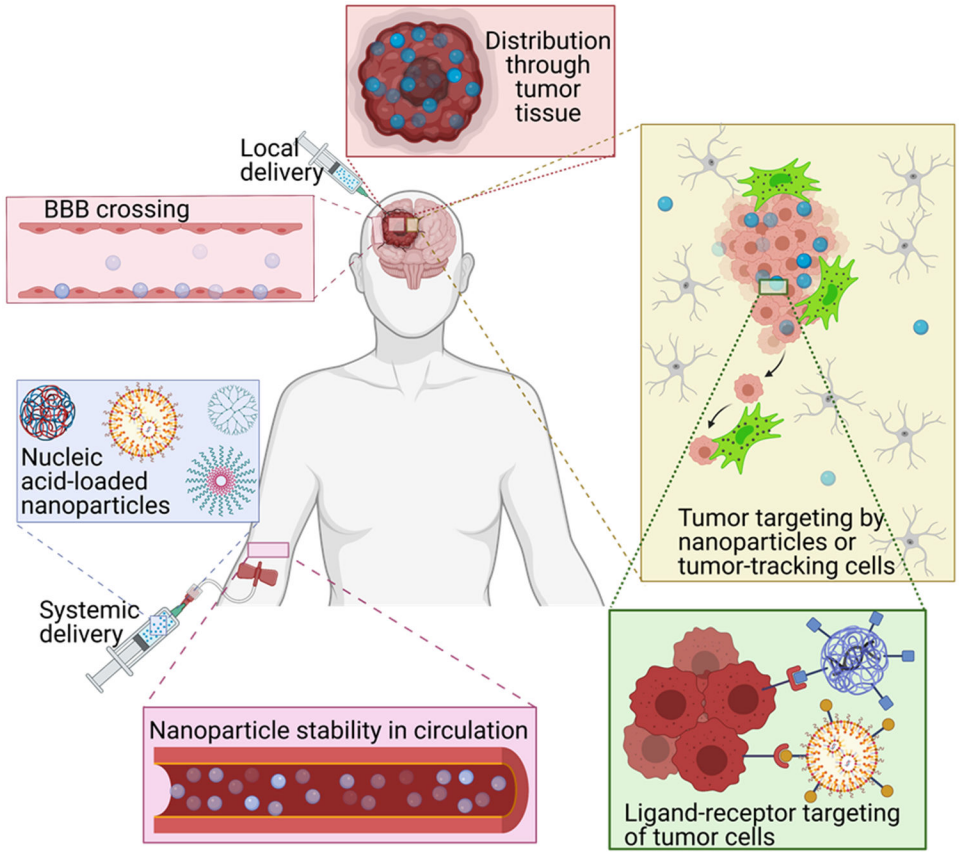


**Figure 1.**

Nanoparticles must overcome multiple barriers for effective gene therapy to the brain. Particles may be administered systemically, after which stability in circulation and extravasation past the BBB into the brain must be optimized, or locally directly at the tumor site in the brain. Once in the brain, nanoparticles must distribute sufficiently throughout the tumor tissue and be taken up by target cells, which may be either tumor cells or surrounding cell types that contribute to tumor growth. This targeting may be accomplished by transfecting tumor-homing cells or by functionalizing the nanoparticles with ligands for receptors on the target cells.

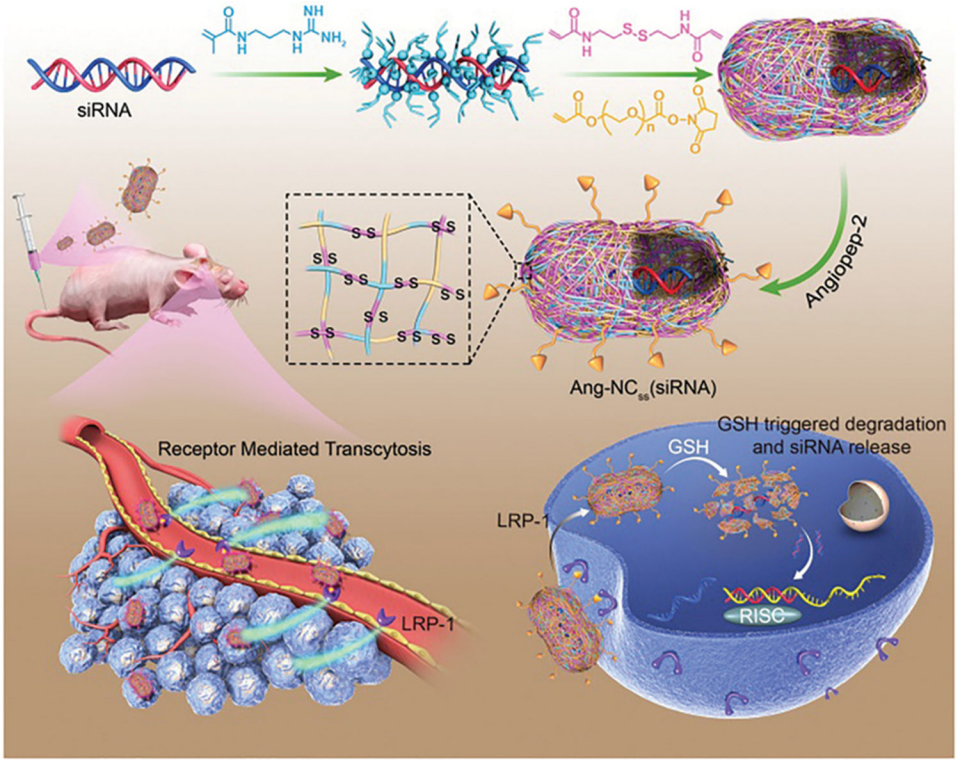


**Figure 2.**

Polymer-based nanocapsules carrying siRNA were functionalized with angiopep-2 [Ang-NC$_{ss}$(siRNA)] for efficient BBB crossing and specific targeting of brain tumor cells. The crosslinks within the nanocapsules contained disulfide bonds for triggered cytosolic siRNA release. Reprinted with permission from Y. Zou, X. Sun, Y. Wang, C. Yan, Y. Liu, J. Li, D. Zhang, M. Zheng, R.S. Chung, B. Shi, Single siRNA Nanocapsules for Effective siRNA Brain Delivery and Glioblastoma Treatment, Adv. Mater. 32 (2020) [78]. Copyright (2020) John Wiley & Sons, Inc.

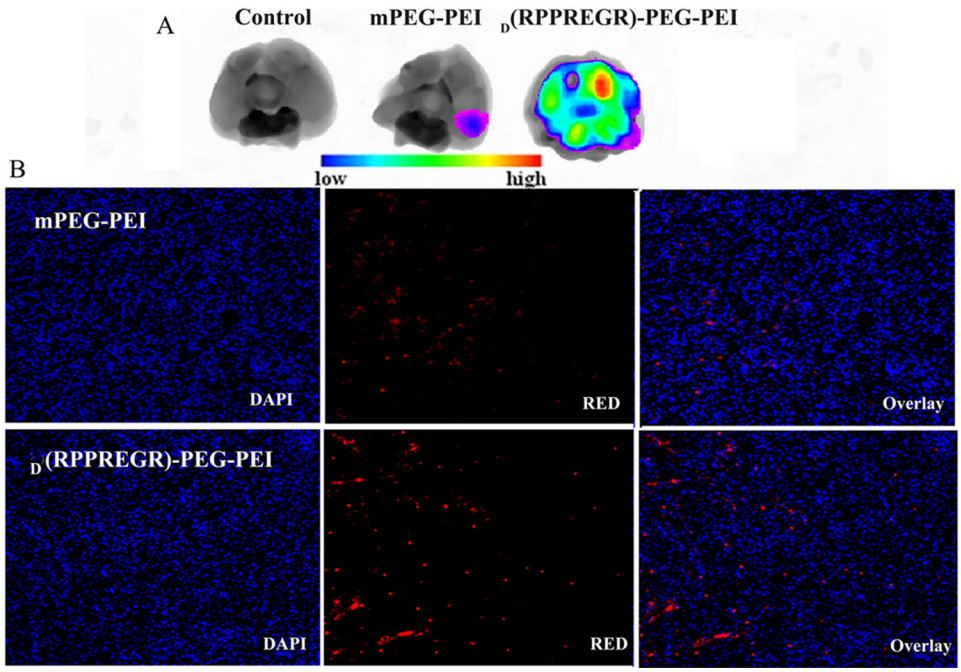


**Figure 3.**

Retro-inverso CendR peptide-functionalized PEG-PEI particles were used to deliver DsRed DNA. DsRed signal in the brain is higher in functionalized particles than in mPEG-PEI particles, measured by fluorescence imaging of the whole brain (A) and fluorescence microscopy on brain sections (B). Reprinted with permission from J. Wang, Y. Lei, C. Xie, W. Lu, E. Wagner, Z. Xie, J. Gao, X. Zhang, Z. Yan, M. Liu, Retro-inverso CendR peptide-mediated polyethyleneimine for intracranial glioblastoma-targeting gene therapy, *Bioconjug. Chem.* 25 (2014) 414–423 [94]. Copyright (2014) American Chemical Society.

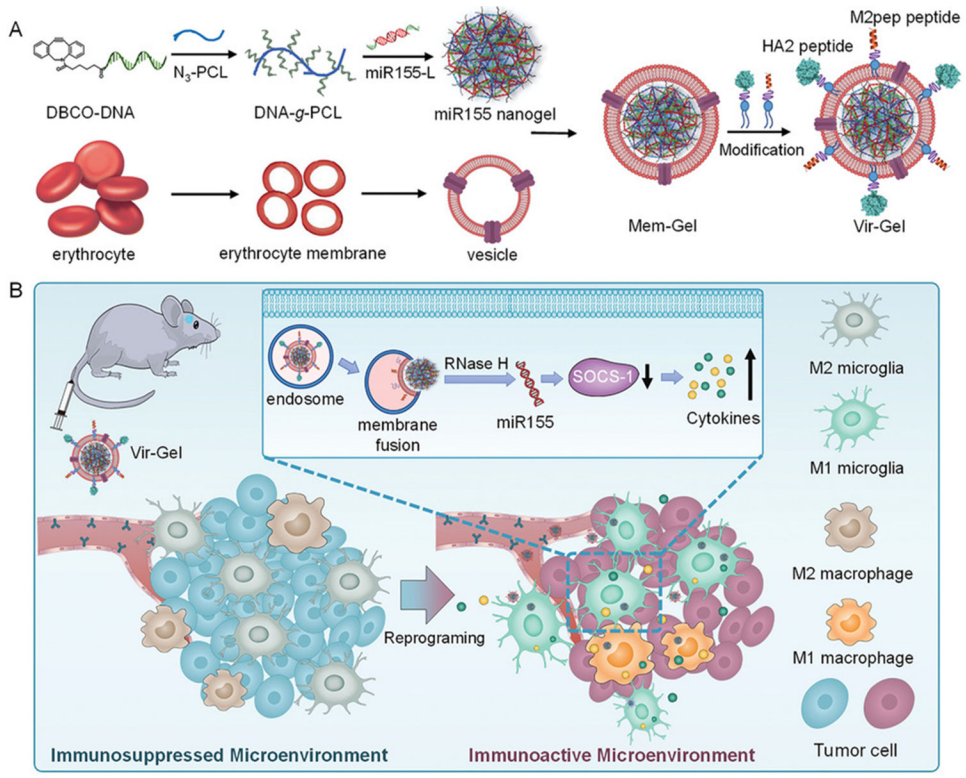


**Figure 4.**

(A) The Vir-Gel is made by crosslinking DNA-grafted PCL with miRNA to form a nanogel, then coating the nanoparticles with erythrocyte membranes and functionalizing with M2 microglia-targeting and membrane fusion-promoting peptides. (B) The functionalized particles reprogram the microglia at the glioma site after administration, resulting in an anti-tumor immune response. Reprinted with permission from X. Gao, S. Li, F. Ding, X. Liu, Y. Wu, J. Li, J. Feng, X. Zhu, C. Zhang, A Virus-Mimicking Nucleic Acid Nanogel Reprograms Microglia and Macrophages for Glioblastoma Therapy, Adv. Mater. 33 (2021) [100]. Copyright (2021) John Wiley & Sons, Inc.

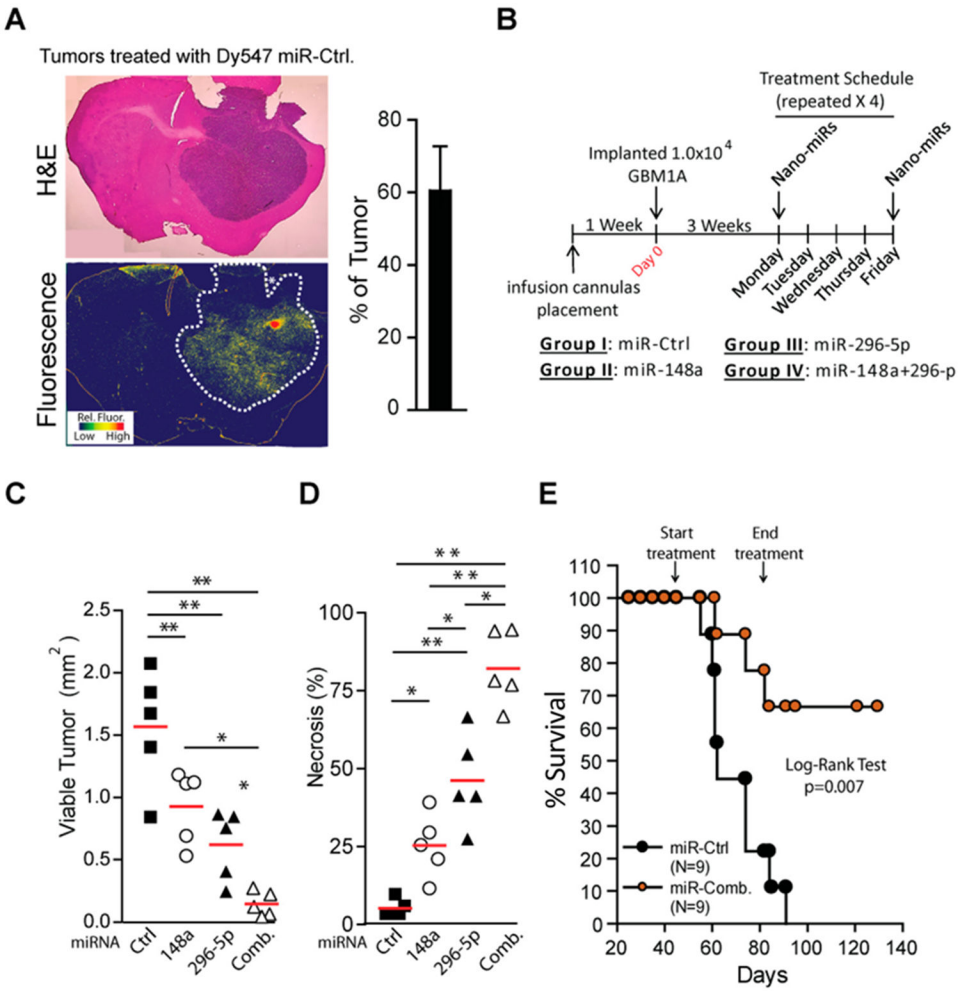


**Figure 5.**

(**A**) The bioreducible nanoparticle formulation (nano-miR) labeled with the fluorescent dye Dy547 was visualized and compared to adjacent H&E-stained sections. The intratumoral distribution of the nano-miRs was about 60% (right panel). (**B**) Schematic summarizing treatment schedule for the *in vivo* delivery of nano-miRs. Animals were sacrificed 42 days after cell implantation to quantify maximum tumor cross-sectional areas showing significantly lower (**C**) viable tumor areas and (**D**) higher necrotic areas for animals treated with nano-miRs compared to controls (Ctrl.). (**E**) miR-148a and miR-296-5p co-delivery using nano-miRs (miR-Comb.) extended the survival compared to mice treated with control nano-miRs (miR-Ctrl). Reprinted with permission from H. Lopez-Bertoni, K.L. Kozielski, Y. Rui, B. Lal, H. Vaughan, D.R. Wilson, N. Mihelson, C.G. Eberhart, J. Laterra, J.J. Green, Bioreducible Polymeric Nanoparticles Containing Multiplexed Cancer Stem Cell Regulating miRNAs Inhibit Glioblastoma Growth and Prolong Survival, *Nano Letters,* 18 (2018) 4086–4094 [118]. Copyright (2018) American Chemical Society.

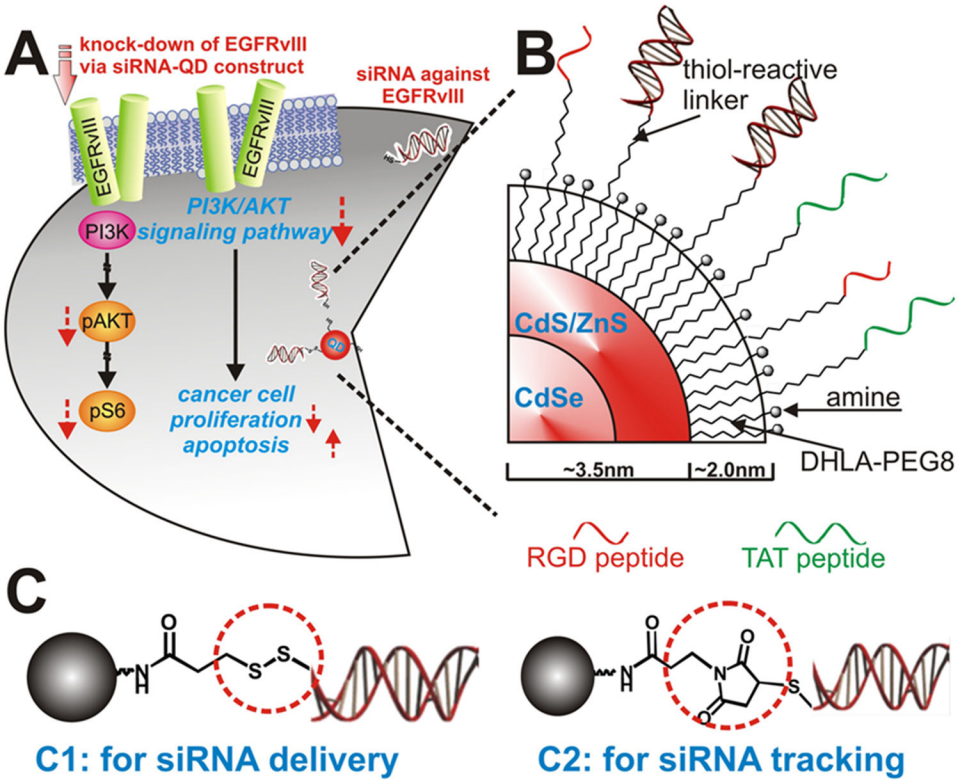


**Figure 6.**

(A-B) Quantum dots were loaded with siRNA targeting EGFRvIII via a disulfide linkage and functionalized with ligands to improve uptake and membrane fusion. (C) The reducible disulfide bridge promotes quick cytosolic release of siRNA, and other linkages can also be used to functionalize the siRNA for intracellular tracking. Reproduced with permission from J. Jung, A. Solanki, K.A. Memoli, K.I. Kamei, H. Kim, M.A. Drahl, L.J. Williams, H.R. Tseng, K. Lee, Selective inhibition of human brain tumor cells through multifunctional quantum-dot-based siRNA delivery, Angew. Chemie. Int. Ed. 49 (2010) 103–107 [147]. Copyright 2010 John Wiley & Sons, Inc.

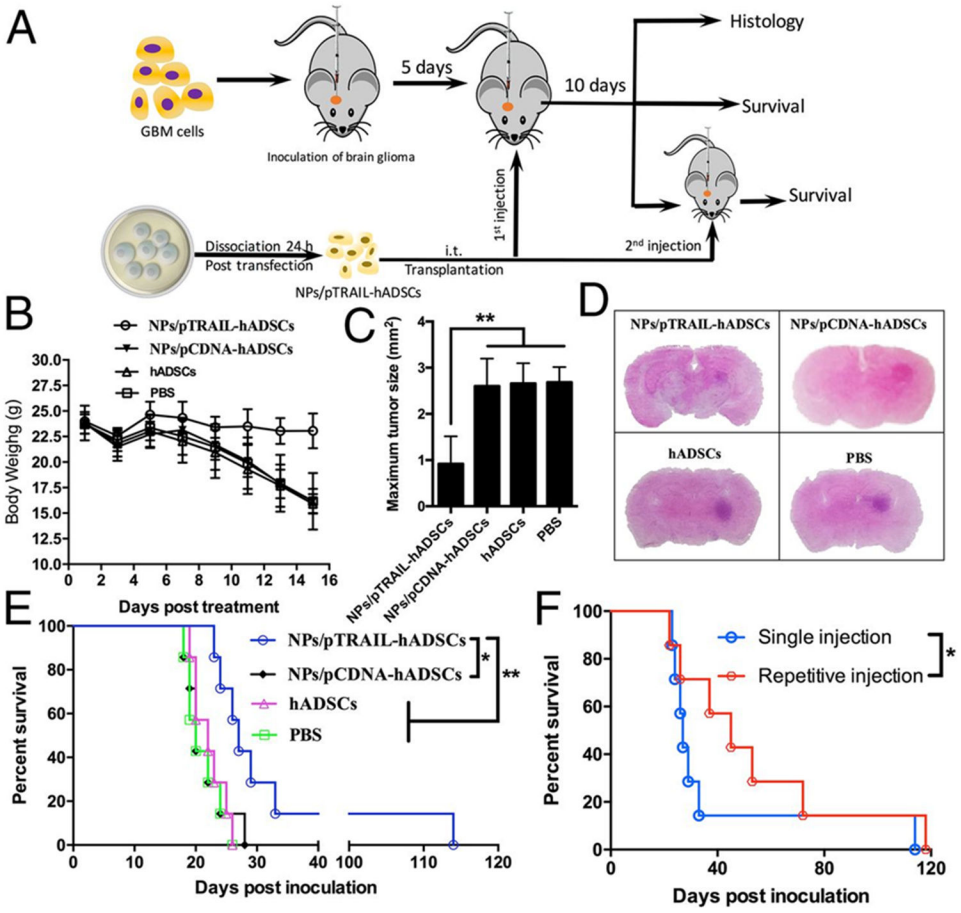


**Figure 7.**

GBM cells were injected intracranially into mice, and ADSCs were transfected *ex vivo* with TRAIL using PBAE-based nanoparticles, then administered to tumor-bearing mice (A). Mice treated with TRAIL nanoparticles fared better, measured by body weight (B), tumor size (C-D), and survival time (E). A single injection of transfected ADSCs had significant survival benefit, but additional dosing also significantly extended median survival. Reproduced with permission from X. Jiang, S. Fitch, C. Wang, C. Wilson, J. Li, G.A. Grant, F. Yang, Nanoparticle engineered TRAIL-overexpressing adipose-derived stem cells target and eradicate glioblastoma via intracranial delivery, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 13857–13862 [185].

**Table 1.**

Nanoparticle Delivery Challenges for Brain Cancer Gene Therapy

| Challenge | Strategies for Overcoming Challenge |
|---|---|
| Nanoparticle or nucleic acid stability in the bloodstream | Nanocarriers to prevent degradation of cargo by nucleases [48] |
| | Control of nanoparticle size to prevent clearance [40,51] |
| | PEGylation to reduce protein adsorption and nanoparticle clearance [54,56] |
| | Crosslinking to reduce surface charge and protein adsorption [52,53] |
| | Surface-functionalization with marker to avoid macrophage uptake [64] |
| | Coating with red blood cell membranes to avoid macrophage uptake [65-67] |
| Crossing the BBB | Ligands for brain endothelium: angiopep-2 [60,63,67,78] |
| | Ligands for brain endothelium: transferrin-targeting [59,61,80] |
| | Ligands for brain endothelium: RGD [90] |
| | Ligands for brain endothelium: targeting glucose transporters [189] |
| | Control of protein corona around NPs [107,190] |
| | Small particle size [78,101,106] |
| | Alternative routes of delivery [110] |
| Biodistribution of nanoparticles within tissue | Convection-enhanced local delivery [119-121] |
| | Control of particle size [118] |
| | Control of particle shape [191] |
| Targeting cell type of interest | Materials-based specificity for cancer cells [119,121,124,125,192] |
| | Glioma-specific ligands: RGD [151] |
| | Glioma-specific ligands: folic acid [128,129] |
| | Glioma-specific ligands: transferrin [81] |
| | Glioma-specific ligands: chlorotoxin [86,87,89] |
| | Glioma-specific ligands: neuropilin-1-targeting [93,94] |
| | Glioma-specific ligands: CD44-targeting [131,132] |
| | Microglia-specific ligands: M2pep [100] |
| | Cancer-specific effect: transcriptional targeting of DNA [148] |
| | Cancer-specific effect: TRAIL [86,151] |
| | Cancer-specific effect: apoptin [153,154] |
| | Cancer-specific effect: knockdown of overexpressed genes [147,155,156] |
| | Cancer-trafficking stem cells as delivery vehicles [185-187] |
| | Transfection of cancer-specific immune cells [165-167,172-176] |
| Endosomal escape into cytoplasm | Membrane fusion-promoting peptides [100] |
| | Proton sponge [118,137] |
| Cargo release from delivery vehicle | Bioreducible linkages [78,118,144-147] |

**Table 2.**

Nucleic Acid Therapeutics and Examples of Their Function for Brain Cancer Therapy

| Nucleic Acid Therapeutic | Intended Function | Intended Therapeutic Target |
|---|---|---|
| DNA | Induce gene expression | Induce apoptosis of GBM cells [81,86] |
| | | Sensitize towards chemotherapy [81] |
| | | Stem cell reprogramming [111] |
| | | Suicidal gene therapy of GBMs [119] |
| mRNA | Induce intracellular protein expression | Cancer vaccine [167] |
| | | Reprogramming of TME [193] |
| siRNA | Silence intracellular protein expression (sequence specific protein) | Inhibit angiogenesis [60] |
| | | Silence immunosuppressive factors [63,90] |
| | | Inhibit proliferation of GBM cells [79] |
| | | Sensitize towards chemotherapy [58] |
| | | Induce apoptosis of GBM cells [101] |
| | | Reprogram brain tumor-initiating cells [121] |
| miRNA | Silence intracellular protein expression (pool of proteins) | Inhibit proliferation of GBM cells [87] |
| | | Reprogramming of TME [100] |
| | | Inhibit stem cell phenotype of GBM [118] |
| CRISPR-Cas9 | Gene knockout | Knockout of oncogenes [120] |