Covalent Nanodelivery Systems for Selective Imaging and Treatment of Brain Tumors

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Abstract

Nanomedicine is a rapidly evolving form of therapy that holds a great promise in superior drug delivery efficiency and therapeutic efficacy than conventional cancer treatment. In this review, we attempt to cover the benefits and the limitations of current nanomedicines with special attention to covalent nanoconjugates for imaging and drug delivery in brain. The improvement in brain tumor treatment remains dismal despite decades of efforts in drug development and patient care. One of the major obstacles in brain cancer treatment is the poor drug delivery efficiency owing to the unique blood-brain-barrier (BBB) in the CNS. Although various anti-cancer agents are available to treat tumors outside of the CNS, the majority fails to cross the BBB. In this regard, nanomedicines have increasingly drawn attention due to their multi-functionality and versatility. Nano-drugs can penetrate BBB and other biological barriers, and selectively accumulate in tumor cells, while concurrently decreasing systemic toxicity.

Graphical abstract

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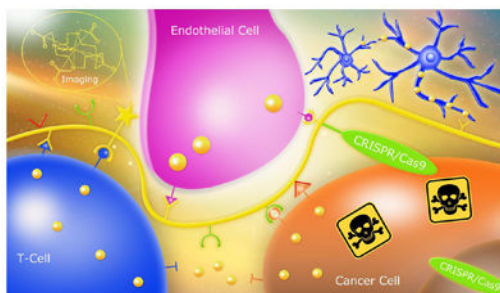
Keywords

Covalent nanoconjugates; Blood-Brain-Barrier (BBB); Nanomedicine for brain imaging; Drug delivery; Brain cancer

Introduction

Medicines have been traditionally given to patients in the forms of pills ("small ball or round mass of medicine", from Middle Dutch or Middle Low German "pille" [1] corresponding to modern encapsulated nanodrugs. Or in the form of a drug as in French "drogue"[2], a natural or synthetic soluble chemical, corresponding to covalent nanodrug. The fundamental difference between pills and drugs is that the active reagent in the pill or capsule is not immobilized by chemical bonds and thus free to evade the carrier material (micelle, liposome, suspended water insoluble precipitate), whereas the drug given e.g., in the form of a soluble nanodrug contains an active natural or synthetic compound, which is covalently bound to a macromolecular platform and often resembles a prodrug.

The main features of a nanodrug are precise targeting and delivery, which are equally important for successful treatment. Addressing safety is of great importance. Unsecured delivery could cause adverse reactions / side effects that occur when a toxic drug and targeted carrier are disconnected during delivery and the drug becomes available elsewhere. To achieve a rapid transport through the body's vascular system, minimize clearance through kidneys and facilitate high penetration through tissue and membrane barriers, imaging or therapeutic delivery vehicles have been developed that cover a range of nanoscale sizes (5-400 nm) [3, 4].

Transported cargo may be both chemically (covalently) bound to the vehicle, and cleaved from the vehicle platform to become the pharmacologically active drug. The bound drugs are not free to diffuse from the carrier, whereas in contrast, encapsulating vehicles transport the drugs in their free unbound form. Encapsulating vehicles are liposomes, micelles, or one of several nanoparticles fabricated by dispersion or precipitation methods. Encapsulating devices release their cargo by spontaneous drug diffusion or after nanoparticle dissolution or capsule erosion. The nano capsules can be designed to "open" at the targeted delivery site in response to the typical environment such as local pH or enzyme cleavage activity. However, because of spontaneous diffusion and capsule-destabilizing environment, release can occur in an uncontrolled fashion and cause harmful damage to healthy tissue. Micelles have a

structure, which is in dynamic equilibrium with their parts forming free constituents. They are self-assembled only when the concentration of the free constituents exceeds the so-called critical micelle concentration (CMC). Below CMC the micelles are instable and dissociate into the free constituents [5, 6], which may occur with injected micelles when they circulate in the vascular system. Concomitantly with the dissociation, the drug located in the micelle core will be released into plasma. Despite this possibility and uncontrolled drug diffusion out of capsules, micelles and other encapsulating devices are frequently used for targeted drug delivery. In this review, we analyze the best possibility for the drug delivery through the multiple bio-barriers with the special attention to the delivery to the brain.

1. Covalent Drug Delivery Systems (CDS)

1.1 Ringsdorf's vision of therapeutic polymers

Ringsdorf proposed the first version of Covalent Delivery Systems (CDS) (Table 1). They became known as "Therapeutic Polymers" because drugs and targeting molecules were attached covalently to a polymer platform [4, 5, 7]. Ringdorf's view was of a linear polymeric nano drug with a central polymer platform: a module with conjugated prodrug binding covalently to the platform by a cleavable linker, and in addition, a module for targeting and a module providing solubility for the therapeutic polymer [8, 9].

1.2 The multi modular architecture of nanoconjugates

This first concept has in the following years been refined to develop polymers that simultaneously carry multiple variable functional groups (modules) in particular, an additional moiety for releasing the therapeutic polymer from endosome vesicle, and aimed at rescuing prodrugs from lysosomal cleavage (Table 1 & Fig. 1). In fact, the only known type of escape until then had been based on the proton sponge effected osmotic disruption of membranes by polycationic devices such as polyethyleneimine, polyhistidine and polylysine for the endosome release of nucleic acids [10]. A decade ago, the tripeptide-based lipophilic endosome escape modules were introduced that could be applied to a large variety of payloads other than nucleic acid [11-14]. Another important module was the antibody for tumor targeting. This novelty allowed the CDS to cross multiple biobarriers including the blood-brain barrier (BBB) [15-22]. Recently, the concept of Mini Nano drugs was introduced that uses receptor affine peptides instead of specific antibodies for targeted drug delivery [23, 24]. In principle, the covalent concept foresees a plethora of modules depending on the number of anchorage sites on the polymer platform. The attached chemically or biologically reactive groups can function synchronously or independently allowing a diversity of reactions in a time and space dependent fashion [4, 5, 7]. Multiple attachments of cell targeting modules offer the possibility to physically connect CD20 receptors to unleash apoptosis in human non-Hodgkin's lymphoma Raji B cells [25] or, as it may be proposed in a scenario with an analogy to special bispecific antibodies (BiTEs), to physically connect epidermal growth factor (EGFR) mutant receptors (EGFRvIII) on tumor cells with CD3 receptor on cytotoxic T-lymphocytes to initiate apoptosis of tumor cells [26, 27].

1.3 Structural and functional diversity of CDS

Because of the macromolecular structure, CDS are versatile molecules. The macromolecular diversity unfolds further when including branched polymers such as dendrimers, molecular rods, and in addition, spontaneous self-association (Table 1) [4, 5, 7]. In the case of a covalently bound drug, these delivery systems should not be confused with nanoparticles encapsulating free drugs.

2. Intramolecular dynamics and group mobility

Depending on their rigidity and status of branching, CDS differ by their ability to exercise intramolecular dynamics. Unbranched nano conjugates differ in rigidity due to the nature of their polymeric units and interconnecting covalent bonds. For example, nano conjugates with polysaccharides, nucleic acids and polypeptides have a stiffer and thus more rod-like structure because of hindered rotation around their backbone. In another example, nano conjugates with polyesters such as poly(acrylic acid), poly(hydroxy alkanoic acid) and poly(malic acid) have backbones interconnected by carbon-carbon and carbon-oxygen bonds. Segments of nanoconjugates are freely rotatable around these bonds. Consequently, spatial crowding inferred by neighboring positions of bulky substituents can be relieved by rotations around the bonds. Furthermore, the rotations contribute to structural flexibility within the platform and could allow spatial clustering of e.g., lipophilic residues [28, 29].

2.1 Antibodies and their replacement by affine peptides

In the absence of antibodies or other large proteins, CDS with free rotatable bonds and extended structures can exercise internal bending motions and coil formation. Bending motions can propel them at higher rates of diffusion than nano conjugates of similar molecular weight but with a spherical shape. Thus, they could achieve faster permeation through membrane barriers and are more effective for deep tissue penetration [30, 31]. Bulky ligands such as antibodies increase size, shape and motion. The rate of interaction of CDS with cell surface receptors is governed by the slow dissociation of the receptor complexes with high affinity binding antibody modules. Their replacement by peptides decreases the size and also binding affinity and accelerates dissociation [32, 33], (Table 2). In our recent preclinical study, the replacement resulted in the reduction of hydrodynamic diameter from 22 nm to 7.8 nm while retaining excellent therapeutic efficacy [23] (Fig. 2).

2.2 Prodrugs and cleavable linkers

CDS are macromolecular entities, which deliver prodrugs conjugated through appropriate linkers. Linkers have been developed that are cleaved in response to exposure to chemistries or enzymatic activities typically found at the site of delivery [34]. Examples include pH-responsive hydrolytic cleavage of activated ester or hydrazides, and the reductive cleavage of disulfide bonds by glutathione in the cytoplasm of recipient cells [4, 35]. Consensus peptide linkers have been introduced that are susceptible to cleavage by specific peptidases such as metalloproteinases MMP-2 or MMP-9 [36], and cathepsins in lysosomes [37, 38].

3. Modular organization of CDS with diverse functions

A CDS is a nano sized macromolecule built from modules specialized in specific functions. One category of modules provides platforms for the chemical assembly of the other modules. Center and "assembly" module is a polymer that provides chemically reactive sites, for example, –OH, -NH$_2$, -COOH, -SH$_2$, -CH=CH-, to attach functional modules. In a wider sense, the "assembly" module includes linkers that could be cleaved for e.g., drug release. Linkers connect the platform with functional modules including drugs, targeting antibodies or peptides, membrane destabilizing molecules or protecting molecules [11-23, 28, 39, 40]. Linkers such as peptides or polyethylene glycol (PEG) can vary in size and flexibility to allow a functional module approach, and interact optimally with its biological target such as a receptor or an enzyme active site, or anneal specifically with a nucleic acid of a given sequence.

Among functioning modules are targeting modules, drug molecules, imaging dyes, contrast agents (MRI), radioactive ^{125}I-labeled tyrosine as tracer molecules, and protective polyethylene glycol (PEG). Targeting modules can be specific antibodies, peptides, oligosaccharides, oligonucleotides or ligands of diverse structures that bind to specific receptors. The targeting ligand must often adopt a certain geometry and orientation to access the specific binding site e.g., pass through a bottleneck at the entrance of a binding pocket or present itself in a conformation that binds a receptor with the highest affinity. An optimal linker should not present geometrical constraints nor unfavorable electrical charges or hydration. A drug conjugated with a linker could be active, or inactive (prodrug). The prodrug can be activated by cleavage of the linker. Converting a prodrug into an active drug is a powerful method for generating high site specificity.

In case a platform offers multiple sites, modules with different specificities can be assigned that function in programmed delivery through several bio barriers to the site of drug action. An example is targeted growth inhibition of lung and breast metastatic tumors in the brain [14]. The designed polymalic acid-based nanodrugs bind with their attached specific antibody to endothelial transferrin receptor (TfR), then pass through the blood-brain barrier (BBB) endothelial cell layer by transferrin receptor-mediated transcytosis. After release from the TfR specific antibody the nanodrugs attach with their Cetuximab or Herceptin antibody to tumor-overexpressed EGFR or HER2 receptors and enter the clathrin-mediated endosome pathway. The nanodrugs remain in the maturing endosomes until the increasing acidification activates the pH-sensitive membranolytic action of the tri-Leucine module [12, 28], and then they finally find their way into the cytoplasm. The pH-responsive membrane disruption is managed by a module consisting of tri-Leucine (LLL) residues conjugated with > 50% of the malyl units of the polymer. After the release of the nano conjugate, cytoplasmic glutathione (GSH) present in > 3 mM concentration reductively cleaves the disulfide containing linker to release EGFR- and HER2-antisense oligonucleotides (AONs) to block EGFR and HER2 specific mRNA. The delivery route and the cleavage of AONs from the polymer platform could be followed by fluorescence of the individually with AlexaFluor 680 and Rhodamine tagged AONs and polymalic acid platform by confocal microscopy. *Ex vivo* western blots showed that the synthesis of EGFR or HER2 and the downstream phosphorylation of Akt were significantly inhibited. PARP was cleaved in

agreement with tumor cell apoptosis [14, 21, 22]. The delivery pathway from nanodrug injection to nanodrug uptake in targeted tumor cells parallels that of imaging enhancement through targeted contrast agents such as fluorescent dyes or MRI contrast agents. The application of brain tumor targeted MRI contrast agents for diagnostic purpose has been demonstrated in tumor bearing mouse models [14].

Covalent nano delivery devices with novel configurations of modules with specific features can open a variety of new avenues: (1) Multiple peptide or chemotherapeutic containing modules in proximal sites could be designed for multivalent binding in the nano and pico concentration range thereby surpassing the binding affinities of antibodies; (2) Linear polymers are polar regarding their termini and could be specifically conjugated at one of them with a (fluorescent) reporter agent. Such devices that could contain a fluorescent terminus could be used in mechanistic studies [41]. Using chemically identical polymer fragments for conjugation at the terminal ends could results in polymer elongation with or without change in direction of polarity, thus opening a new direction of synthetic approaches.

Due to the multiplicity and spatially precise attachment points, covalent delivery devices offer a variety of underexplored possibilities not only in controlled drug delivery but also in controlled interconnections between receptors on single cells and those on different cells or on arrays of cells, especially on immune system cells.

4. CDS structural coherence

Covalent nano delivery systems are macromolecular entities displaying coherence of all residues. This "covalent coherence" distinguishes CDS from "association driven coherence" of non-covalent nano carriers (NCDS).

Covalent coherence is controlled by a precise composition and structure. Although a biological CDS preparation is not monodisperse, the principle of coherence is maintained. This does not hold for NCDS. Solvent precipitation based nanoparticles slowly dissolve, and in the case of micelles nanoparticles follow self-association ruled by the CMC [6]. In these cases, the polydispersity reflects different polymer contents, and the particle surface constantly reforms due to the particle-internal mobility of the constituents. The structural interface to the solvent may depend on internal mobility, composition and chemistry of the constituents, solvent composition, the temperature and the time of circulation in the blood. Then, a serious consequence is the uncontrolled release of drug from the delivering nanoparticle e.g., by diffusion. Diffusion depends on chemical composition and physics as well as on size, shape and chemistry of the drug. Release could be extreme for hydrophilic and low molecular chemotherapeutics and could give rise to toxic side effects especially during prolonged treatment. Attempts to minimize the diffusion have involved crosslinking between the nano capsule forming components. However, diffusion can remain substantial especially during long-term circulation of nanoparticle. A typical example for uncontrolled *in vitro* release into plasma from nanoparticles manufactured from poly(D,L-lactide-co-glycolide) (PLGA)-poly(ethylene glycol)(PEG) has been reported for docetaxel (DTX) indicating a 50% release over circulation time [42].

5. Examples of Covalent Nanodelivery Systems based on polymer platform

5.1 Poly(β-ʟ-malic acid)

Polymalic acid of high molecular weight was discovered by its ability to mimic nucleic acids and competitively inhibit DNA polymerase α of *Physarum polycephalum*, and, to a lesser extent, the activities of DNA polymerases from other organisms [43, 44] (Fig. 3). Later on, poly(β-ʟ-malic acid) (PMLA) was shown to have a functionality as a highly suitable platform for successful covalent delivery system due to an easy chemical substitution chemistry, biodegradability, absence of toxicity, and absence of immunogenicity. In preclinical studies, it was proven as an optimal platform to deliver chemotherapeutics such as doxorubicin and temozolomide, as well as antisense oligonucleotides of the Morpholino type to solid human breast cancer, human brain tumor, and metastases [11-23, 28, 39, 40]. The maximum tolerated dose of poly (β-ʟ-malic acid) is 1.0 g/Kg [19]. Polymalic acid is available by synthetic chemistry in its D,L racemic form and in structural variants involving the carboxylic group in α- or β-position of the malic acid building unit and in unbranched/ branched variants (summarized in [45]). Nature-made PMLA is an unbranched polymer of the β-L-isomer [11, 43, 46, 47]. Molecular weights are 5-10 kDa when isolated from *Aureobasidium* and other fungal strains [46, 48, 49] and 30-300 kDa when isolated from the plasmodium, the vegetative cell form of *Physarum polycephalum*, and from other members of the myxomycetes clad [46]. Polymalic acid has pendant carboxylic acid groups, which are ionized at physiological pH and render the polymer highly soluble [45]. Approximately 50% of the carboxylate groups may be derivatized with leucine ethyl ester or tri-Leucine without notably affecting the solubility in serum [50]. Polymalic acid-based drug delivery systems called "Polycefins" [11], have been synthesized with highly purified pharmaceutical quality PMLA from the *Physarum polycephalum* isolate [13-15, 17-22, 39, 40] (Fig. 1A), whereas synthetic PMLA was also used for the synthesis of other conjugates [51]. Intravenously injected PMLA-based nanodrugs are delivered through the tumor vascular endothelium by conjugation to antibodies that recognize TfR overexpressed in tumor capillary system [11-14, 17-22, 39, 40]. Receptor binding is followed by endothelial transcytosis through BBB into brain tumors and subsequently into tumor cells receptor mediated endocytosis. Tumor cell uptake is targeted by antibodies against TfR, EGFR and HER2 in the case of glioblastoma and brain metastases of human triple negative breast tumor, and metastases of HER2-positive human breast cancer, as well as other metastatic tumors in the brain [12, 14]. The highly specific recognition and delivery to brain was demonstrated for animals containing simultaneously different brain tumors by applying MRI contrast agents equipped with the tumor specific targeting antibodies and which were otherwise the same as the nanodrugs used for treatment [14]. The successfully delivered antisense oligonucleotides specifically blocked mRNA synthesis of overexpressed HER2 and EGFR/EGFRvIII in the tumor cells achieving significant prolongation of tumor bearing animal survival [11-14, 17-22, 39, 40].

5.2 Poly(L-amino acid)s

Anionic polymers structurally related to PMLA are homo polymers of aspartic (PAA) and glutamic (PGA) acid. Peptide bonds unlike ester bonds typically impose stiffness of the polymer backbone in contrast with high rotational flexibility of the ester backbone in

polymalic acid. This restricts spatial orientations of covalently attached bulky groups such as proteins or oligonucleotides, and impairs intra and inter polymer interactions between intrinsic and extrinsic ligands.

The pendant carboxylic groups of these homopolymers are fully ionized at neutral pH giving rise to polyanions with slightly lower charge densities as for PMLA. Similarly, these polymers can occur in two structurally linear variants, one involving the carboxyl group in α-position (e.g., α-PGA), and the other one involving the carboxyl group in γ-position (γ-PGA) next to the amino group. Branched variants are possible when both types are verified in a single polymer molecule. α-PGA and γ-PGA are naturally synthesized. Low molecular weight α-PGA has been obtained by recombinant technology; however, the production is problematic, whereas chemical synthesis by nucleophile-initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid is feasible [52].

In comparison with PMLA, the peptide bonds of polyamino acids are relatively stable against spontaneous hydrolysis at neutral and moderately acidic pH. In human, α-PGA is systemically degraded by specific peptidases. Efficient degradation of γ-PGA after injection into humans is disputed, and it may not be cleaved by specific peptidases. When tested on cell culture level, maximum tolerated concentrations varied between 10 mg/kg and 100 mg/kg. At a dose of 1 mg/kg injected into mice intravenously, γ-PGA was well tolerated. The γ-PGA amide bonds are cleaved by extracellular peptidases of *Bacillus subtilis* and of other *Bacillus* strains [52-54]. Although mammals lack appropriate peptidases, γ-PGA was found to be edible. Efficient γ-PGA uptake into blood from the intestines or clearance through feces remain to be conclusively demonstrated. Cleavage by stomach hydrochloric acid and by the intestinal microbes [55] can occur during food digestion and may involve specific γ-glutamyl transferases [56] in mammals [57].

5.3 Poly(α-L-glutamic acid)

Poly(α-L-glutamic acid) (α-PGA) has a respectable history as a covalent drug delivery system. α-PGA platforms have been reported for the delivery of paclitaxel (TXL), camptothecin, and of other chemotherapeutics [58-60]. "Passive" delivery by the Enhanced Permeability and Retention (EPR) effect (tumor-compromised enhanced permeability of vasculature and lack of lymphatic drainage) has been highly effective in therapy with paclitaxel and camptothecin [58-60] without the need to involve specific receptors for active tumor targeting. The high tumor specificity has been suggested to depend on M2 macrophage uptake of α-PGA nano drugs before infiltrating tumors (reviewed in [58]). The chemotherapeutics were conjugated forming pH-labile responsive ester bonds with the γ-COOH of glutamic acid units [58, 59]. Release catalyzed through peptide bond cleavage by lysosomal cathepsin B ([61] and reviewed in [58]) has been considered as another pathway of paclitaxel activation and exit from endosomal compartments into the cytoplasm. Active targeting of TXL- α-PGA conjugate was achieved using cyclic RGD, c(RGDfK), or the dimer glutamate-c(RGDfK)$_2$ targeting modules which specifically bind $\alpha_v\beta_3$ integrin receptor overexpressed on tumor endothelial and epithelial cells. Because the integrin targeted TXL- α-PGA conjugates have hydrodynamic diameters of 7-8 nm. They can be considered as "mini nano drugs" and reminiscent of the PMLA-based mini nano drug [23]

but distinguished from most manufactured anti-tumor nano drugs of sizes 30-200 nm. Mini nano drugs are considered as providing improved anti-tumor activity through high diffusion rate and permeability. Delivery of paclitaxel, camptothecin, doxorubicin and other anti-tumor chemotherapeutics by conjugation with α-PGA and their use in clinical trials Phase I-III has been reviewed [58]. Paclitaxel α-PGA conjugate (Polyglumex) has entered clinical trials as radiation sensitizer in temozolomide radiation therapy of glioblastoma [62].

Polyglutamic acid is successfully used as conjugation platform for covalent attachment of gadolinium DTPA- and DO3A-complexes and of NIR813 fluorescent dye functioning in single and dual MRI combined fluorescence imaging (reviewed in [58]). As suggested for treatment, high uptake efficacy by certain tumors was noted, which supported the M2 macrophage uptake mechanism in favor of α-PGA specificity to target tumors. In the study of delivery efficacy, it was noticed that overly sustained blood circulation of α-PGA gadolinium contrast agents raised safety concerns. To accelerate clearance, α-PGA-cystamine-Gd(III)-DOTA conjugate was introduced, which had a short residing time [63]. However, because of the strong EPR effect, α-PGA-gadolinium contrast agents might not be suitable for tumor type diagnosis by clinical MRI.

5.4 Poly(γ-(DL)-glutamic acid)

γ-PGA is synthesized by a variety of microorganisms including cultured *Bacillus subtilis* (reviewed in [53, 54, 64]. The capsules of virulent strains of *Bacillus anthracis* contain solely γ-D-PGA [65] protecting the bacterial cells against phage infections and also preventing antibody recognition and uptake by macrophages [64, 66]. Naturally occurring γ-D-PGA is nontoxic, however, in combination with two other secreted factors the polymer becomes the dangerous *Bacillus anthracis* exotoxin [67, 68]. Although the *B. anthrax* capsule is not immunogenic, the poly(D-γ-glutamic acid) component or fragments could become immunogenic when conjugated to peptides or proteins. The highest levels of IgG anti-γ-D-PGA were elicited by decamers of γ-D-PGA at 10–20 mol per unspecified protein bound to the N- or C-terminal end [69]. Given these data, immunogenicity of γ-D-PGA oligomers or polymers could be of concern if any peptides and protein were bound to γ-D-PGA-nano conjugates for drug delivery.

In contrast to ribosomal synthesis of poly(α-L-glutamic acid), poly(γ-L(D)glutamic acid) is synthesized by a membrane-bound protein complex in bacteria [52, 64]. Polymers of high molecular weight $\sim 10^5 - 8 \times 10^6$ Da can be isolated. Natural γ-PGA contains a mixture of L-glutamic acid and D-glutamic acid [64]. D-glutamic acid from *B. anthracis* and similar γ-(DL)PGA from other microorganisms take a helical conformation when in the unionized form, and a varying random coil conformation with increased ionization [53, 64, 70]. In contrast, several conformations are reported for α-PGA including α-helix, β-sheet and random coil at varying pH and salt concentrations [53, 64]. Unlike α-PGA, the γ-PGA backbone characteristically contains periodically repeated hydrophobic - $(CH_2)_2$ - alkyl segments that provoke hydrophobic interaction and aggregation, especially when pendant carboxylic groups of the polymer chain are neutralized.

γ-PGA shares many of the pharmacological properties of α-PGA, such as unusually effective passive (EPR) tumor targeting and long blood circulation after intravenous

administration. Although γ-PGA has multiple pendant carboxylates for conjugation of chemotherapeutic and oligomeric nucleic acid drugs, it has not been used much for drug delivery in contrast to the multiple applications of PMLA, which also has a multiplicity of chemically reactive pendant carboxylic acid groups (see above). One of the reasons is that the hydrophobic nature of γ-PGA renders nano conjugates less water soluble with a high tendency for self-aggregation. Anti-breast tumor activity has been demonstrated for cis-platinum(II) compounds coordinated with inter chain and intra chain γ-PGA carboxyl groups [71]. Both PGA α- and γ-isomers may be applied for nucleic acid delivery because of their stabilization of nucleic acid complexes with polycations through hydrophobic interactions, or as nucleic acid binding conjugates with cationic ligands. The PGA is supposed to facilitate uptake and reduce cytotoxicity [72, 73]; however, nucleic acid delivering nanoparticles on the PGA basis that could target tissue or cells have not been forwarded to clinics. The reason for this could be the difficulty of chemical ligation of PGA and nucleic acids in overcoming the electrostatic repulsion between the negatively charged molecules. If this is the problem, it could be overcome by conjugating of PGA with neutral polynucleotide versions such as phosphodiamidate morpholino oligomers as demonstrated for conjugates with PMLA.

5.5 Poly(aspartic acid)

Similarly to poly(malic acid) and poly(glutamic acid), poly(aspartic acid) exists in D,L stereoisomers, structural α- and β- isomers and branched α,β variants. Synthesis and biodegradability of PAA have been reviewed [74]. Structurally, poly(α,β-(D,L)-aspartic acid) and poly(α,β-(D,L)-malic acid) are similar except the replacement of the amido group in PAA by the ester group in PMLA. Remarkably, the spatial distances of polymer pendant carboxylates are similar, which was recognized in experiments measuring the inhibition of DNA polymerases by polyanions of variable structures [44]. In this study, a similarity of the distance between the negatively charged carboxylates in PMLA and PAA with phosphate groups in nucleic acids has been noted. In another study where the degree of membrane destabilization by copolymers of poly(β-L-malic acid), poly(α-aspartic acid), poly(α-glutamic acid) and polyacrylic acid was measured, it was found that poly(β-L-malic acid) and poly(α-aspartic acid) had the highest membrane leakage activities [50]. These observations could inspire the designs of new PAA-based nano conjugates for drug delivery. To date, the following interesting results have been reported. (1) Hydrogels for drug delivery: an injectable hydrogel was obtained by hydrazone formation of aldehyde-modified PAA, which released cargo in response to late endosome and lysosomal pH [75]. In another version, poly(ethylene glycol) monomethyl ether (mPEG) and doxorubicin were conjugated onto polyasparihyazide (PAHy), prepared by hydrazinolysis of polysuccinimide, and formed effective anti-tumor nanoparticles of approximately 200 nm size suitable for pH-responsive delivery of doxorubicin [76]. (2) Poly(aspartic acid) segments have been shown to bind to hydroxyapatite (HA) and could be applied for the targeting of nanoparticles to bone tissue [77]. (3) Because of its biocompatibility including biodegradability and negligible immunogenicity, the potency of PAA to form different covalent combinations with polycations and cyclodextrin to form nucleic acid complexes for gene delivery have been recognized and developed into biodegradable nucleic acid delivery assemblies [78, 79]. In these studies, PAA was employed as the platform for covalent attachment of several kinds of

nucleic acid interactive molecules, such as ethylamine derivatives to form polycation-like structures, benzyl alcohol esterification of pendant carboxyl groups to introduce hydrophobicity, and cyclodextrin for engaging host-guest nucleic acid interactions. Despite the structural similarity with PMLA, macromolecular (all covalent) conjugates of PAA have not been used as delivery vehicles. In contrast, the manufacture of micelles or of composites in combinations with nucleic acids and chemotherapeutics is preferred, which would allow stabilization of nucleic acid delivery vehicles through the addition of electrostatic interactions between polyanionic polyaspartate, various polycations, and hydrophobic polymers [80, 81].

5.6 Polyacrylic acid and its derivative N-(2-hydroxypropyl)methacrylamide copolymer (HPMA)

Polyacrylic acid (PAA) as a pH-responsive polymer, poly(propylacrylic acid) (PPAA) as a pH-responsive membrane destabilizing polymer [82], and especially N-(2-hydroxypropyl)methacryl amide copolymer (Fig. 1) combining pH responsivity, membrane destabilization activity, and ability for covalent drug delivery [83], are a group of polymers evolving from the favorable polymerization chemistry of acrylic acid. HPMA was introduced by the Kopecek laboratory in Prague during the early 1970s after appreciating its favorable relationship between hydrophilicity and biocompatibility, in particular the stable chemistry of the N-substituted methacrylamide group [84]. The PAA is not biodegradable due to the lack of enzymes that could cleave the C-C bonds of the polymer backbone. Moreover, because of their high molecular weights, HPMA and conjugates lack renal clearance. Longevity of such conjugates could be harmful with a risk of storage diseases. By interspacing short PHMA copolymer stretches permissive to renal clearance with peptides that are substrates of peptidolytic enzymes, such as papain or lysosomal cathepsin B, HPMA copolymers have been redesigned to be "biodegradable" [85-87]. HPMA copolymer has been explored into various applications to covalently attach or deliver a broad variety of chemotherapeutics, saccharides, peptides, and antibodies for specific targeting of cells and tissues [88], including chemotherapeutics that were membrane permeable but poorly soluble in aqueous media. Such chemotherapeutics are conjugated via a cathepsin B cleavable tetrapeptide linker GFLG or by a pH-sensitive hydrazone linker [89]. These drugs could escape the lysosomal compartment by their autonomous membrane permeation activity when cleaved from the HPMA conjugate. Several of these HPMA drug conjugates have entered clinical trials [90].

Among the variety of conjugates, an interesting variant was constructed, which colligated single cell surface CD20 antigens of human non-Hodgkin's lymphoma (NHL) Raji B cells by binding to multivalent HPMA copolymer-Fab′ conjugates resulting in the induction of apoptosis [25]. In a further study, crosslinking was initiated by annealing of complementary morpholino antisense oligonucleotides (AON) located on independent Fab' conjugates (a novel drug-free nanotherapeutic treatment of B-cell malignancies) [91]. However, this approach of complementary annealing of morpholino AON on HPMA to trigger cell death was not translated into using AON to block mRNA unlike the AON-PMLA conjugates that demonstrated impressive antitumor activity [13, 14, 17-22, 39, 40]. In an alternative approach, HPMA carrying triplex forming oligonucleotides (TFOs) were used for treating

liver fibrosis by inhibiting the transcription of α1(I) collagen gene [92]. The 25-mer fully phosphorothioated oligomer TFO-3'-NH2 was conjugated with nitrophenylester-activated Poly (HPMA-co-GFLG-ONP) at the C-terminus of the cathepsin B specific peptide [93]. The α-D-mannopyranoside containing nano conjugate was targeted to hepatic stellate cells (HSCs). Type I collagen gene expression was significantly inhibited when HSC-T6 cells were transfected with this conjugate following tail vein injection into rats. In another approach, HPMA copolymer contained N-(2-(2-pyridyldithio)ethyl)methacryl amide. In a thioldisulfide exchange reaction miRNA was covalently attached to PHMA via the formed reducible disulfide linker [94, 95]. Whereas the delivery of covalent HPMA nucleic acid conjugates parallels the delivery of morpholino AON in PMLA-based nano drugs, the more classical approach of the delivery of nucleic acids involving noncovalent attachment through electrostatic anchorage has been reported on a large scale [96-98]. The HPMA conjugated cationic stretches were oligolysine (12-mers or lower) and oligolysine stretches attached to oligonucleotides [96-103]. These constructs bind and compact DNA with variable length tailored to the size of HPMA-oligolysine copolymers [102]. To reduce toxicity, the constructs contained either cathepsin-cleavable oligopeptides or reducible disulfide linkers that were cleaved by lysosomal peptidase or reductively in cytoplasm. To manage the escape from endosomes, the constructs carried histidine residues to induce proton sponge osmotic rupture of the endosomal membrane or contained lipophilic compounds for lytic membrane destabilization [103]. Novel syntheses based on covalent attachments to HPMA offer great advantage over the application of other polyanions due to their easy and controllable reversible-addition fragmentation chain-transfer (RAFT) polymerization, which has been well advanced [7, 100, 103, 104]. Amino acids and peptide derivatives containing a terminal vinyl group are readily copolymerized into strategically opportune positions in these constructs [60]. Beside the development of excellent nano drugs, HPMA copolymers have been designed for *in vivo* tumor tracking and imaging using various imaging agents [105-107] or radioactive isotopes [87], a recent example being using the IR-783 dye for uMUC-1-targeted near infrared colonoscopy[107].

6. Coherence of polymeric nano drug design

6.1 Hydrodynamic shape and size

The shape of a CDS in solution, whether rod, coil or sphere, depends on chemical design. Elongated and rod-like CDS have been built by chemical conjugation of modules with sites of functional groups distributed along unbranched polymers such as *N*-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) or PMLA, PGA, dextran, and chitosan. The effect of substitution on CDS shape has hitherto not received much attention. Polymalic acid-based CDS will be considered here as an example.

The biologically produced PMLA is a polyester between the hydroxyl group and the β-carboxylic group of L-malic acid leaving the α-carboxylic group pendant position and reactive for substitutions activity [13, 14, 17-22, 39, 40]. Activated as N-hydroxy succinimide (NHS) ester the carboxyl group reacts with free amino groups as part of simple linkers, peptides and proteins. Linkers containing mercapto groups serve to form thioethers and disulfide substitutions including morpholino AON. Nearest neighbor effects on the

substitution by hydrophobic residues such as leucine ethyl ester result in hydrophobic clusters that are responsible for membrane destabilization and permeation [28]. Studies of unsubstituted PMLA in distilled water by cryo-transmission electron microscopy indicated open, coiled forms and double stranded structures between non-ionized stretches of the polymer (unpublished). Calculation based on the chemical structure indicates the length of the polymer strand (50 kDa) of approximately 80 nm that is reconcilable with the length of the observed double stranded structures. Consistent with coiling is the hydrodynamic diameter of 4-7 nm measured by dynamic light scattering for PMLA of 50-100 kDa. After various degrees of substitutions with PEG (5%, percent denotes the fraction of malyl units conjugated with the ligand), AONs (1-4%), antibodies (1-5 molecules per polymer), and peptides as leucine ethyl ester or trileucine (40%), the nanoconjugates remain highly soluble and are biologically active. Hydrodynamic diameter measured as a function of molecular weight increased in a biphasic fashion with a break at the transition from free to substituted PMLA [19]. This biphasic behavior is interpreted by the assumption of a polymer coil in phosphate-buffered saline (PBS, pH 7.4) with the size indicated at the transition. When the polymer is conjugated with increasing number of modules, the particle size expands indicated by the second phase of hydrodynamic diameter as a function of molecular weight [19].

6.2 High axial ratio in support of CDS environmental interactions and directional mobility

CDS based on unbranched polymer platforms display their targeting modules and cargo (drug) along the platform polymer. By the interaction with solvents and functional ligands the coil can unfold to an extended configuration. In this state the nano conjugate has a high length to diameter ratio with a geometry that favors a high number of environmental contacts, e.g., of bound ligands with receptors, access to reagents and enzymes for linker cleavage and to receptors in control of degradation and clearance. A high axial ratio supports directionality of movement in the direction of the long axis, and also suggests that thermally induced bending motions propel the polymer through densely packed environment. The successful delivery of AON by a "streamlined" PMLA-based mini nanodrug is in agreement with the proposed mechanism [23].

6.3 Coherence and biodegradability

Another result of coherence is propensity for biodegradation. Biodegradation into fragments is usually carried out by enzymes starting cleavage from one of the termini (exo enzymes) or after recognizing signature sequences located within a polymer (endo enzymes). Biologically derived CDS are biodegradable. Well known examples are proteins, nucleic acids, carbohydrates. Whereas there is no problem with recognizing endolytic cleavage sites in CDS, such sites buried in micelles or solvent precipitation/evaporation manufactured particles are accessible only after nanoparticle dissolution. Biodegradable polymeric platforms are highly desirable in drug delivery systems precluding toxic storage diseases, immunological responses or toxic side effects due to unresolved degradation and systemic clearance. In case of HPMA, which contains exclusively non-biodegradable aliphatic C-C interchain bonds, an elegant solution has been invented by periodically placing short non-immunogenic peptides into HPMA using click chemistry [85, 86]. The short HPMA fragments remaining after cleavage by cathepsin B can be exported from the organism

through renal clearance. Targeted peptidolytic cleavage has found application in tumor MR imaging and tumor targeting by cleavage at peptidase signature sites [108, 109].

Nature-made polymers are usually biodegradable in mammals. Examples used in nanomedicine are PMLA, α-PGA, γ-PGA, and chitosan. Unsubstituted PMLA is also degraded by spontaneous hydrolytic ester cleavage via oligomeric intermediates to L-malic acid (half-life related to molecular weight is10 hrs at pH 7.4, 37°C [110, 111]). The rate of spontaneous hydrolytic cleavage is considerably decreased after substitution at the pendant α-carboxylic groups of the polymer. A polymalate depolymerizing enzyme is secreted by *Physarum polycephalum* [112, 113], and enzymes have been identified in bacteria [114], fungi [46], and as serum lipases in human [115]. The malic acid generating activity of the *Physarum* PMLA hydrolase is stalled at branching points and pendant substitutions. Poly(L-glutamic acid) is cleaved by cathepsin B highly expressed in tumor lysosomes, but with no measurable activity in serum, allowing long-lived covalent conjugates of paclitaxel with exclusive activation of paclitaxel in tumor lysosomes [116]. Enzymes that cleave y-glutamyl linkages have been identified and characterized in mammals [117, 118], fungi [119], bacteria, and phages[120, 121]. Chitosan is degraded by chitosanases (EC 3.2.1.132), and products have been well characterized [122].

During treatment, injected CDS is spontaneously or enzymatically cleaved exolytically from one of the polymer ends by depolymerases yielding free polymer building units, or in most other cases endolytically forming fragments that may contain both targeting and pharmacologically active modules which are still prospective nano drugs. Fragments devoid of prodrugs may target receptors, acting as competitive inhibitors of biological or regulatory pathway(s). The kind of fragments containing only prodrugs will not develop side effects due to lack of cell uptake and drug activation. With time, degradation continues towards clearance through kidneys, liver, spleen and macrophages. In contrast, cleavage sites of NCDS are often buried inside the nanoparticles. But without cleavage, active drugs can be released as a result of spontaneous carrier dissolution due to change in ionic strength, pH, temperature, or by detergents and other solubilizing agents. Free drugs, acidification and carrier degradation products can give rise to systemic toxicity.

6.4 Examples of modules with specific functions

The central module of CDS is the polymer platform, which outlines the particle shape and dynamic flexibility. The platform carries all modules with functions to achieve optimal delivery (Fig. 1B): receptor targeting, cleavage from the delivery system and destabilizing of membranes. Linkers combine functional modules with the platform. They can be responsive to enzyme activity, pH or redox reactions outside or inside recipient cells. Other modules such as protective PEG, fluorescent dyes and MRI contrast agents (e.g., Gadolinium-DOTA) can be attached to the platform with the purpose of inhibiting enzymatic degradation, systemic clearance by macrophages, liver, spleen, or as means for local tracing of CDS, and MRI diagnostics.

6.5 Platforms, typical chemical/structural outfit

Covalent nano delivery systems are typically unbranched linear polymers, and branched typically spherical polymers (e.g., dendrimers). The polymer is built from repeating units, which in the case of linear CDS can be designed to carry functional groups along the polymer and in the case of dendrimers, functional groups in terminal positions of the branches. Examples of repeating units are amino acids such as glutamic acid and aspartic acid, or hydroxycarboxylic acids such as malic acid and citric acid, or saccharides such as glucose and a glucose amine. In principle, each unit can be chemically conjugated with specific functional ligands resulting in a plethora of CDS that cannot be matched by NCDS.

6.6 Platforms for Precision Molecular Therapy and Imaging

Using gene sequencing, molecular cancer markers can be identified within a short period of time after diagnosis. Using the antisense technology, gene expression can be efficiently silenced by clinical treatment with antisense modalities such as siRNA or AON. When using CDS with multiple attachments, one or several genes can be simultaneously targeted by the same platform delivering at the same time different AON or other drugs. The delivery and simultaneous multiple knock down has been demonstrated with a set of AON against EGFR, HER2, CK2, laminin α4 and laminin β1 genes [13, 14, 17-22, 39, 40].

7. Prodrugs

When drugs are covalently bound to CDS, they become inactive, i.e. "prodrugs". Conjugation requires a chemical "handle" on the drug, which is removed from the drug during the release reaction or remains part of the cleavage product without interference with its pharmaceutical action. An example is the 3'-thiopropylamido group of AON that does not adversely affect annealing with the target mRNA. Examples of complete chemotherapeutic drug reconstitution are pH-responsive hydrolysis of 2'-carboxylic acid esters of paclitaxel and docetaxel or of hydrazone derivatives of doxorubicin, summarized in [35]. Examples of incomplete removal of linker components include the cleavage of linker peptides by lysosomal cathepsin B in the case of doxorubicin [38, 123] or reductive cleavage of disulfide linker by the cytoplasmic glutathione in the case of AON [11, 12]. Cleavable linkers have been recently reviewed [35].

8. Nucleic acids delivery by covalent nanodrugs

Nucleic acids have been permanently conjugated through peptides or reversibly through cleavable disulfide linkers. Two principle antisense variants are available: nucleic acids such as siRNA carrying negatively charged phosphodiester bonds, and neutrally charged synthetic variants replacing natural ribose phosphate backbone by neutrally charge amido phosphate morpholino residues. Whereas both variants anneal perfectly with template nucleic acids, the neutral version is invulnerable to nucleolytic activity and is synthetically easy to conjugate with polyanions such as PMLA. In addition, the morpholino variant behaves invariantly to most peptides and proteins when assembled on delivery platforms [33, 124]. Routinely used 25-mer morpholino AON are below renal threshold and have been reported as nontoxic. Because of morpholino AON, targeted nanomedicines with PMLA conjugates have been

very successful in inhibiting tumor synthesis of molecular cancer markers such as laminin α4 and β1 chains, EGFR/EGFRvIII, and HER2 [11-14, 17-22, 39, 40].

9. Covalent Nanodelivery Systems for Imaging and treatment of Brain Tumors

9.1 Nanomedicine Overview

Based on their structural properties, nanodrugs can be divided into the following categories: liposomes, dendrimers, polymer conjugates, micelles, carbon nanotubes, gold nanoparticles and magnetic nanoparticles [125]. Due to their unparalleled payload capacity, versatile targeting, and multi-functional properties, nanomedicines can be equipped with almost all types of moieties such as chemotherapy, radioactive agents, nucleotide oligomers, antibodies, proteins or peptides as well as imaging agents for therapeutic or diagnostic purposes. More importantly, nanodrugs can significantly increase the drug bioavailability while decreasing its side effects via specific biological barrier penetration mechanisms as well as drug targeting mechanisms [126, 127]. Here, we briefly summarize the major nanostructures listed in Table 3 with an emphasis on their application to the brain diseases. A human brain is structurally unique and complex relative to other organs in the existence of multiple biological barriers both inside and outside the brain tissue [128]. Therefore, drug delivery to the brain has always been the most challenging task in drug development. In the context of brain delivery, each category of the nanostructure has its own strength and limitation as a drug carrier, and these structural features impose both challenges and opportunities in their application in drug development, especially in CNS diseases. Covalent nano conjugates with their chemical stability in plasma and superior tissue targetability are attractive drug candidates for the penetration of multiple biobarriers, delivery of the active moieties into the brain cells, and release without intermediate neurotoxic subproducts.

9.2 Brain Barriers: Blood-Brain-Barrier

A human brain is highly vascularized but is also selectively isolated from other parts of the body by the blood-brain-barrier (BBB). Described as the "bottleneck in brain drug development," the BBB consists of brain endothelium held together by tight junctions that forms a barrier around the brain with highly selective permeability [135]. In addition to the endothelial cells, other participating cells lend even more regulation to this barrier, including astrocytes, pericytes, microglia, and the vascular basement membrane [132]. For example, astrocytes surround the BBB with their end-feet, secreting factors that upregulate tight junction proteins and express key enzymes and transporters that contribute to the proper functioning of the barrier [132]. Microglial cells are an important part of brain's immune defense system, and they are promptly activated and able to swallow pathogens and present antigens for T cell activation when the foreign entities pass through BBB and invade brain environment [136]. Pericytes have been shown to play a critical role in regulating BBB permeability as well as the integration of other cell types [137].

Importantly, the BBB functions to maintain a constant environment inside the brain by regulating cerebral extracellular fluid and protecting the brain from environmental noise; but when pharmacological treatment is needed, the same barrier prevents the extravasation of

most drugs useful for treatment of CNS diseases such as brain tumors [14, 134, 138]. The BBB excludes most polar molecules, but certain nutrients and ions can gain access through highly regulated receptors [139]. Although the BBB has been observed to be altered at the tumor core (termed the "brain-tumor barrier", or BTB) with somewhat disrupted selectivity (Fig. 4), it is intact adjacent to the BTB, allowing peripheral malignant cells to evade treatment [140]. Some possible mechanisms for BBB penetration include passive diffusion, active transport by transcytosis/endocytosis, or by inhibiting efflux pumps (Fig 5 [140]). Furthermore, other physiological obstacles limit progress in brain cancer treatment, as will be described later in this review. We are going to use the term "BBB" instead the "BTB" formally used in the literature when speaking about brain tumors. We would like to emphasize the fact that despite the known differences between normal and tumor endothelial/vascular systems, they still represent similar obstacles for the drug delivery and treatment of brain cancer.

9.3 Brain Barriers: Blood-CSF Barrier (BCSFB)

The Blood-CSF Barrier (BCSFB) is formed by the epithelial cells of the choroid plexi and other circumventricular organs (CVO) [141]. Different from BBB, the capillary cells of the BCSFB do not have tight junctions, and they are fenestrated allowing for free permeation of various substances [142]. Circumscribing these capillaries are polarized epithelial cells with tight junctions, preventing free diffusion between blood and surrounding CSF. The choroid plexus produces CSF and regulates the movement of solutes between blood and CSF [141, 143]. Reservoirs of CSF exist in the subarachnoid space and the ventricles, exchanging fluid and solutes with surrounding tissue [143]. CSF cycles bidirectionally through brain parenchyma by mixing with the interstitial fluid and then reentering the reservoirs mentioned above [143]. The exchange of substances through this cycling is essential for maintaining homeostasis in the brain, and the integral membrane proteins aquaporins are critical for this movement of fluid. Three pathways have been outlined for drug delivery involving CSF, including blood borne (drug moves from CSF to blood and then to brain through BBB), diffusion (drug in CSF diffuses to brain through BCSFB), and convection (drug moves from CSF to brain through bulk flow of perivascular spaces), but these mechanisms have shown very limited effects with current methods [128]. Future research into the BCSFB may provide novel insight into delivery mechanisms. However, in exploring these mechanisms one must be aware that drug penetration into CSF cannot be used as a measure for BBB permeability, as they are completely distinct barriers with different characteristics. It is also essential to understand that drugs injected into the CSF are unlikely to reach deep brain parenchyma; novel methods of delivery are needed in order to exploit this pathway for therapeutic purposes [128].

9.4. Brain Cancers

Brain cancers, both primary and metastatic, are the leading cause of cancer-related deaths in the United States in patients below the age of 35 [144]. It is widely accepted that gliomas are among the most hostile tumors – with the most dangerous being Glioblastoma Multiforme (GBM) – and result in significant morbidity and mortality [138, 145, 146]. GBM is a Grade IV astrocytoma per WHO classification system for CNS tumors [147]. At the genetic level, GBM tumors can be divided into four molecular subtypes with distinct genetic signatures,

termed Neuro, Proneuro, Mesenchymal and Classical subtypes [148]. Due to the nature of GBM, tumor recurrence is extremely common even with the most aggressive treatment regimens including surgery, radiotherapy, chemotherapy and even hemispherectomies [138, 144]. Physicians and scientists have attempted to tackle this aggressive cancer from all angles, employing innovative surgical and therapeutic techniques to curb the progression of this disease. However, improvement in the outcome of GBM patients has remained dismal in the past 25 years. Today, the standard of care in brain tumor treatment consists of cytoreductive surgery, followed by chemotherapy [140, 144, 149, 150]. Along with radiation, Temozolomide (TMZ) continues to be the first line chemotherapy since its first approval for GBM treatment by FDA. Whereas new treatments in other fields of medicine continue to improve remarkably, the little progress made in brain cancer treatment and diagnosis is due to the highly aggressive nature of brain tumors as well as to the inability of many therapeutic agents to overcome the biological barriers associated with the brain.

In 2016, a four-year initiative, National "Cancer Moonshot", was launched with a $1 billion funding to jump start this program [151]. For reasons such as this, the near future holds great promise for innovation in the field of brain cancer treatment. This review will discuss the obstacles in brain cancer diagnosis and treatment, in addition to the potential application of nanomedicine. We will cover important considerations for the design of an ideal nanotherapeutic or imaging agent, and outline promising future directions of nanomedicine in the context of brain tumor diagnosis and treatment. Specific attention will be given to covalent nanomedicines (covalent nano conjugates) as stable in plasma and promising for the BBB delivery.

10 Application of Nanotechnology for Brain Cancer Imaging

When treating a brain tumor, the first step is its proper diagnosis, e.g., through imaging. Ideally, before a suitable course of treatment is prescribed, a physician needs to properly identify the grade, histological type, genomic markers, location, and origin of the tumor. Historically, tumor biopsies have been the major source of this information. However, unlike the situation in most organs, brain biopsies carry a certain danger for the patient with 6-12% complications, and are in some cases impossible to perform [Malone et al. (2015), World Neurosurgery]. For this reason, imaging, with its many different modalities, holds potential as a non-invasive technique to assess these parameters in the brain. Furthermore, imaging helps physicians determine whether surgical intervention is necessary [152].

With the rapid technological advancement, imaging has grown significantly from a once solely anatomical tool to the one that now assesses a range of different biological and morphological signals, making for a highly powerful diagnostic approach. Anatomy-based imaging of the past allowed for the evaluation of structural abnormality and tumor-related complications [153]. With the implementation of modern imaging techniques, it is now possible to read functional, hemodynamic, metabolic, cellular, and cytoarchitectural abnormalities [153, 154]. This continuing innovation now allows imaging to be used to diagnose and grade tumors, and to accurately monitor patient prognosis, all without physically breaching the brain.

Below, we will briefly cover the major modalities commonly used in brain imaging, which are summarized in Table 4.

Nanotechnology offers many advantages in medical applications and has received tremendous focus in both the therapeutic and diagnostic settings. The goal has been to employ nanotechnology to create clearer images for more accurate diagnosis and treatment monitoring, while keeping non-specific toxicity to a minimum. The following sections will describe the impact nanotechnology has had on brain tumor imaging, and its applications in precision medicine.

Currently, many imaging modalities are used either alone or in combination to improve diagnostic capabilities. In nano imaging, the two main approaches are MRI and fluorescent imaging [156]. Nonetheless, other traditional imaging modalities such as CT, PET, and ultrasound are commonly engaged for the imaging of brain tumors.

10.1 Magnetic Resonance Imaging (MRI)

Currently, Gadolinium (Gd)-enhanced MRI is the leading imaging modality for brain tumor diagnosis, owing to Gd's inherently large magnetic moment [157]. Although Gd is a highly toxic substance to humans, its chelated form is both safe and able to maintain its important paramagnetic properties when it is acting as a contrast-enhancing agent [158]. In the context of brain tumors, Gd-enhanced contrast increases the differentiation between tumor and brain tissue, allowing for visualization of a more defined tumor border [152]. However, there are limitations in traditional Gd-contrast agents: they have poor accumulation and retention within the brain. Furthermore, there are severe limitations in their imaging power: they are unable to determine the etiology of brain tumors; they are not able to distinguish between primary brain tumors and metastasized brain tumors; and they are unable to identify tiny early-phase metastatic lesions [159]. As a result, Gd-based nanoparticles have been formulated to achieve higher targeted Gd accumulation, longer retention within the brain, and stronger T1 contrast (due to higher accumulation), potentially allowing for the identification of tiny lesions [160]. Multiple Gd^{3+}-based contrast nano formulations have been clinically accepted [161]. Recently, MR-guided focused ultrasound (MRgFUS), which is a non-invasive procedure that reversibly opens the BBB without damage to the surrounding neurons, has been employed for targeted drug delivery to the brain involving non-human primates. Results of a clinical trial designed to establish the feasibility, safety and preliminary efficacy of MRgFUS to open the BBB for the delivery of chemotherapeutic agents in brain tumors has been approved by Health Canada. Preliminary data have shown an increased Gd uptake in the sonicated area of the brain [162].

Recently, a landmark paper was published by Patil et al. in which the "MRI Virtual Biopsy" method was introduced [14]. This innovative method allows for the differential diagnosis of lung and breast brain metastatic tumors, the two most common primary cancer sites for brain metastasis. HER2 and EGFR antibodies were covalently conjugated to a CDS with PMLA backbone that also contained one or two imaging agents: chelated Gd for MR imaging, and/or Alexa Fluor 680 for optical imaging. This multi-modal imaging concept allowed for the confirmation of the MRI results by means of fluorescent imaging. The used nano imaging agents were able to preferentially accumulate in different tumors in the same brain

tissue based on differences in molecular markers, allowing for non-invasive tumor typing helpful for subsequent therapy (Fig. 6).

Though Gd is generally safe in its chelated form, Gd-induced nephrogenic systemic fibrosis (NSF) has been reported. It is believed that a small amount of Gd may de-chelate while in circulation inducing NSF [158]. Therefore, alternatives to Gd-based nano contrast agents have been investigated. The most common non-Gd-based MR contrast agents are based around iron-oxide. In 1978, Ohgushi et al. discovered that iron-oxide possessed powerful inherent contrast properties for T2 imaging [163]. As a matter of fact, iron-oxide-based nanoparticles were the first to be used for imaging ([164, 165]). In the context of brain tumor imaging, current clinical trials are focusing on Super-Paramagnetic Iron Oxide Nanoparticles (SPIONs) for the detection of microscopic metastatic lesions and enhancing the contrast of malignant brain tumors [164]. Today, multiple SPIONs are used in clinic: for example, Feridex/Endorem, and Resovist.

10.2 Fluorescent Imaging

Due to its non-invasive, highly sensitive, real-time imaging capabilities, optical imaging has become one of the most popular imaging modalities in nanomedicine. However, when applying it to the *in vivo* systems, problems such as autofluorescence and peripheral absorption arise [166]. These problems prevent deep-tissue imaging, as many biological entities act as fluorophores of particular wavelengths. Consequently, the poor penetration depth of many optical agents is a hindrance to their use. In response to the autofluorescence of endogenous biological structures, scientists have discovered that minimal interference occurs when exciting fluorophores with near-infrared (NIR) wavelengths [166]. Due to their potential cytotoxic effects, NIR-emitting dyes are still under investigation. They come in two major forms, but the most commonly employed *in vivo* forms are various synthetic fluorophores. A recent study by Kim et al. used glioma-bearing rats that were administered mesenchymal stem cells (MSCs) tagged with synthetic fluorophore-labeled nanoparticles. This group was the first to reveal that *in vivo* NIR imaging could be used to track the distribution of injected MSCs [167]. Importantly, no significant differences (e.g. cytotoxicity) were seen between labeled and non-labeled MSCs.

The second major type of NIR fluorophore is based on Quantum Dots (QDs). Due to their easily modifiable surfaces and high resistance to photobleaching, diverse QD fluorescent nanoparticles have been constructed. QDs usually contain elements from metal groups II, III, and V of the periodic table, as their electrons are loosely bound to the atom, allowing for their excitation and subsequent photon emission [168]. QDs are ideal agents for crossing the BBB, as they are small (<10 nm in diameter) and modifiable with homing moieties [166, 169]. Currently, QDs are still under investigation for their potential cytotoxic profiles. To minimize toxicity, QDs can be incorporated into nontoxic carriers such as dendrimers and liposomes [170, 171]. Despite their potential hazardous side effects, QDs hold a great promise for the future of brain cancer treatment. They may be very helpful in demarcating tumor boundaries for intraoperative detection, as fluorescent imaging is capable of highlighting clearly defined tumor borders [172, 173].

10.3 Computed Tomography (CT)

Advancements in nanotechnology have also facilitated improvements within the realm of CT. Due to its low cost and ability to quickly generate images, CT scans are commonly used today. However, even with the use of traditional iodine-based contrast agents, CT scans have severe drawbacks, including rapid renal clearance coupled with strong renal toxicity. Iodine-based contrast nano agents have been developed, improving both the retention and toxicity issues [166]. These contrast agents, which can be used for effective tumor detection, are based on high atomic number elements such as iodine, gold, and Gd [174, 175]. In particular, gold-based CT contrast nano agents have garnered attention recently due to their high X-ray absorption coefficient, easily modifiable surface, and biocompatibility [166]. Though nano-related CT imaging for brain tumor visualization is not explored well, some important findings were published recently. For example, Hainfeld et al. created a gold-based nanoparticle for brain tumor radiotherapy and CT imaging [176]. Due to high electron density of gold, these nanoparticles were visualized by high-resolution CT, which showed a 19:1 increased uptake between brain tumor tissue and normal tissue, respectively.

10.4 Positron Emission Tomography (PET)

In addition to MRI and CT, PET also serves as an important tool for imaging. Distinct from traditional MRI and CT, PET scans are functional, allowing for metabolic assessments on a molecular level. PET has multiple useful cancer applications, including important drug-delivery measures such as biodistribution and pharmacokinetics [177], defining the "actual" tumor volume [178], and differentiating between metabolically active and post-treatment necrotic tumor masses [179]. However, due to its lack of structural anatomical information, PET alone is not a preferred imaging technique for cancer diagnosis. Instead, PET coupled with CT or MRI is often used, as these dual-imaging techniques reveal both anatomical and functional molecular information. Though PET/CT is the traditional form of dual-modal imaging, PET/MRI has recently gained popularity in cancer imaging, as certain types of cancers (e.g., brain, head, neck) are better visualized by MRI than by CT [180]. For example, a study demonstrated that radioisotope-bearing nano micelles can be constructed and used for the treatment and imaging of rat GBMs. To assess certain parameters such as anatomy, accumulation, biodistribution, and local blood volume, PET/MRI dual-modal imaging was applied [181]. However, few studies have been published so far involving nano agents in PET-based imaging for brain tumors.

10.5 Ultrasound (US)

US scans are commonly employed in imaging because of their high safety, real-time imaging capabilities, and low cost. Currently, a drawback of US is its poor contrast resolution; even with the use of microbubble contrast agents, the resolution is not as high as with other imaging modalities [164]. Moreover, microbubbles are too large to readily extravasate into tumors, thus limiting the application of US for tumor imaging. Due to these limitations, perfluorocarbon-based nanodroplets have been developed, which are capable of entering tumors [164]. However, these nanodroplets have poorer contrast than the more traditional microbubbles, which is a major deterrent for use in tumor imaging [182]. Luke et al. recently reported "super-resolution ultrasound imaging" of a new generation of nano-

sized contrast agents, laser-activated nanodroplets (LANDs), which seem to hold future promise for deep tissue molecular imaging [183]. Further investigations need to be conducted to explore the application of LANDs to brain tumor targeting.

Unlike other imaging modalities, US also serves a vital functional role outside of imaging. In particular, focused ultrasound (FUS) has become an important technique for drug delivery across the BBB (Fig. 7), with reported sub-millimeter precision [184]. In a recent study, Chen et al. synthesized TGF-β1-conjugated microbubbles to facilitate the extravasation of nanoparticles into mouse glioma cells with the help of FUS [185]. When FUS was applied, the microbubbles physically disturbed the BBB, rendering it more permeable. Furthermore, application of FUS allowed the microbubbles to release TGF-β1, which further disturbed the BBB by reducing pericyte wrapping of the endothelial cells. It was determined that, in the presence of microbubbles, FUS facilitated nanoparticle extravasation into the glioma cells [185].

11. Brain Cancer Treatment

11.1 Primary Brain Cancer Treatment

Gliomas are the most common and aggressive primary brain tumors [145, 186]. They can be divided into four malignancy levels according to WHO grading: low grade tumors, grades I (pilocytic astrocytoma) and II (diffuse astrocytoma), are characterized by faster-than-normal proliferation and a possibility of spreading to nearby tissues [147]; high-grade tumors, grades III (anaplastic astrocytoma) and IV (glioblastoma multiforme), are characterized by increased cell and blood vessel density, atypical cells, necrosis, and high mitotic and metastatic activity [147, 186]. Statistically, 10% of glioblastomas are secondary neoplasms; the remaining 90% are *de novo* GBMs, which have a rapid onset of only 3 months (compared to 4-5 years with secondary glioblastomas) [145]. Very limited options have been made available for treating primary brain cancer in recent history: from the 1960s until 1998, only two drugs were approved for the treatment of brain tumors [187]. In 1999, Temozolomide (TMZ), a DNA alkylator, was approved for treating primary brain tumors and remains the standard of care today ([150, 188]. Chemotherapy for recurrent GBM is very limited and usually results in little if any benefit. For instance, Carmustine (BCNU) wafers are widely used for treating glioblastoma, however, many studies showed that BCNU treatment did not increase patient survival or the quality of patients' life [189, 190]. A small fraction of GBM patients showed promising response to tyrosine kinase inhibition, such as to EGFR inhibitors. However, the elevated PI3K/AKT activity in GBM tumors often diminishes the therapeutic advantage of EGFR inhibition [191]. Despite collective effort of physicians and the scientific community, the median post-diagnostic survival of patients with GBM remains 13-16 months on average [145, 147].

11.2 Secondary Brain Tumor Treatment

Brain metastases (BM) are a common and severe complication of other cancers, and they present even stronger challenges to treatment due to their significant morbidity. The most common origin of BM includes cancers arising from the lung, breast, colon, kidney, and skin [192]. BM are present in up to 40%-50% of metastatic lung cancers and 25% of metastatic

breast cancers, with autopsy revealing about twice as many cases [193, 194]. Progress in primary breast cancer treatment has led to an increase in patient longevity but has also increased the chance of residual cells metastasizing, in particular to the brain [153]. Even lower than that of primary brain tumors, the median survival of patients with BM is only under 6 months [194].

Pharmacological treatments in the clinic for primary breast and lung cancers commonly include their respective therapeutic monoclonal antibodies, such as trastuzumab and cetuximab. Trastuzumab, which targets a receptor tyrosine kinase HER2, an EGFR family member, and cetuximab, which targets EGFR, are effective for treatment of certain primary breast and lung cancers, respectively (Fig. 8, [14]). However, like most drugs, they cannot penetrate the BBB to reach brain tumors on their own, and thus fail to treat BM [153].

12. Designing a Nanodrug for Treating Brain Tumors

The primary objective of a chemotherapeutic drug is to be able to treat cancerous tissue specifically. However, most traditional drugs produce non-specific toxicity, causing unwanted damage to healthy tissue. As will be described in this section, one of the major advantages of nanomedicine is that it allows for homing modifications and for selective targeting. Thus, nanotherapeutic agents (NTs) are viable candidates for the treatment of many diseases, including brain tumors, as the sites of interest can be specifically targeted to reduce general toxicity [195]. However, to produce an effective NT, many factors must be taken into consideration.

12.1 Choosing an appropriate nanocarrier

Depending on the purpose of the NT, and the region in which it will act, different nanocarriers are chosen. There are pros and cons to using any type of nanocarrier, and in Table 3, we summarized commonly used BBB-crossing nanostructures with their advantages and disadvantages, respectively. The size, shape, surface properties and mechanical stiffness (termed as 4S Parameters) of the nanodrugs are particularly critical for their transportation and stability in the circulation, tumor-specific accumulation as well as clearance [196]. In all these areas, certain preferences do exist. For example, in the treatment of CNS diseases, lipid-based formulations are preferred due to their hydrophobic nature, which may allow for passive diffusion across cell membranes [197]. However, it is important not to limit designs according to these preferences. Promising *in vivo* results have been obtained in brain tumor research by using non-lipid-based nano formulations. In one of such examples, our work successfully demonstrated BBB permeation in mice using a non-toxic, biodegradable nanobiopolymer, PMLA, as the nanocarrier for delivering GBM therapeutics (Fig. 9, [14, 19, 198]).

12.2 Optimizing Circulation Time

For a therapy through systemic delivery to work, the drug has to stay in the circulation for an optimal period of time. One of the advantages of nanomedicines is the increased circulation time relative to traditional treatments [199]. Yet, even with NTs, circulation can be cut short by reticulo-endothelial system (RES) clearance [200]. When highly hydrophobic molecules

form aggregates with hydrophobic plasma proteins, blood clots form. The formation of these clots triggers phagocytic RES macrophages to remove the source of the problem, which is the hydrophobic molecule [200, 201]. Thus, to evade macrophage clearance, NTs are commonly modified with hydrophilic conjugates, such as PEG [199]. By adding a hydrophilic stabilizer, protein aggregation is prevented, thus removing the trigger that initiates macrophage-dependent clearance [202].

For many years, PEG has been the go-to for macrophage evasion. Currently, there are over 10 FDA-approved PEGylated nanomedicines, and more than 20 are in clinical trials [203]. In the context of CNS delivery, it has been widely reported that PEG does not prevent BBB crossing. However, PEG influence on BBB-crossing mechanisms has not been extensively researched [204]. Furthermore, PEG may cause other side effects; for example, PEG can induce an immune response, initiating the production of anti-PEG IgM antibodies [205]. These anti-PEG antibodies allow macrophages to quickly recognize PEGylated molecules upon re-exposure, thus facilitating RES clearance [205]. Nonetheless, PEGylated nanomedicines remain the golden standard today.

In addition to RES clearance, NTs can also be removed by the kidneys, though this is not nearly as significant of an obstacle. Renal clearance is far easier to overcome than RES clearance, due to its strict size dependence: if the particle's hydrodynamic diameter is less than 6 nm, it will undergo rapid renal clearance [206]. Thus, renal clearance could easily be avoided by increasing the size of NT [206].

It is important to note that there is an optimal circulation time: an NT should remain in the body long enough to produce an effect, but not so long that it starts to cause problems. Prolonged drug accumulation could potentially cause toxicity. Importantly, it has been demonstrated that the biodistribution of drug conjugates could be tuned based on their physicochemical properties. For example, Hamilton et al. demonstrated that larger particles had higher accumulation across virtually every tissue in the body, whereas the smaller particles were more readily excreted [207].

12.3 Targeting and Crossing the Blood-Brain Barrier

One of the biggest obstacles in the treatment of brain tumors and other CNS diseases is bypassing the BBB [197]. The BBB is exceptionally particular in the material it allows to cross from blood to brain. Studies have shown that the BBB prevents 100% of large molecules and 98% of small molecules from diffusing freely across the barrier [208]. However, nanomedicine often exploits two phenomena that allow for NTs to selectively accumulate in the tumor mass: the EPR effect and BBB-receptor targeting.

As previously mentioned, one of the most exciting advantages of nanomedicine is its ability to limit general toxicity through specific tissue targeting. Typically, the development of a brain tumor triggers abnormal angiogenesis, as tumors have an imbalance of proangiogenic and antiangiogenic factors [202, 209]. As a result of the disordered angiogenesis, "leaky" blood vessels with compromised structural integrity are formed, increasing BBB permeability in certain areas of the tumor vasculature [202]. This well-known phenomenon, the EPR effect, is the passive mechanism by which small, polar NTs can selectively enter

brain tumors [210]. However, the effect is minimal compared to a more active form of targeting, as the EPR effect is limited to the small areas of the BBB that are impaired (Fig. 5).

Distinct from the passive targeting mechanism, NTs are capable of actively targeting tumors, further facilitating their accumulation in the tumor cells [211]. This ability is conferred to NTs through the conjugation of specific targeting moieties. Importantly, this ability can only be exploited because different physiological structures express distinctive biomarkers [199]: for instance, malignant endothelial cells overexpress the TfR, as they require a larger amount of iron [211, 212]. This overexpression allows NTs to actively bypass the strict constraints imposed by the BBB: by carrying transferrin-targeting moieties, such as transferrin peptides, NTs can target and bind to endogenous BBB TfRs [213]. Once the NTs engage their targeted receptors, they are shuttled into the tumor mass via receptor-mediated endothelial transcytosis [214]. In addition to receptor overexpression by malignant cells, certain receptors are highly concentrated in specific areas of the BBB, allowing for selective drug delivery [215]. For example, Tumor Necrosis Factor (TNF) transporters are highly expressed near the hypothalamus - this locally increased receptor expression would allow TNF-targeting NTs to specifically target hypothalamic gliomas [216].

The two mechanisms described above – EPR and receptor targeting – are the most common ones seen in NT BBB crossing. Other less common mechanisms worth mentioning include adsorption-mediated endocytosis and carrier-mediated transport [217]. Furthermore, physical manipulations such as FUS and hyperthermia have been also employed for BBB penetration (Fig. 7) [208, 218, 219].

12.4 Penetrating the Brain Tumor

As described in the previous section, various mechanisms may be employed to direct NTs into brain tumors. Yet, there is still an important hurdle to navigate once the NTs enter the tumor: the tumor vasculature. The abnormal angiogenesis responsible for the EPR effect also results in the formation of irregular blood vessel networks. Due to this vascular abnormality, blood vessels are not as uniformly dispersed throughout tumors as they are throughout healthy tissue. An extreme example of this irregularity is displayed throughout GBM vasculature [209, 220]. Therefore, the perfusion of drugs from blood to tumor is heterogeneous, resulting in large pockets within the tumor mass that cannot be easily accessed by the NTs [221]. One promising strategy to mitigate this issue can be an anti-angiogenic treatment, potentially normalizing the vasculature and permitting the drug to better penetrate the tumor [209, 222].

However, fixing the irregular blood vessel network would not completely solve the diffusion problem: once inside the tumor mass, the NT must also be able to travel through the extracellular matrix (ECM). Diffusion through the tumor ECM is regulated by collagen grids, which are highly upregulated in glioma ECM [223]. Low-collagen tumors facilitate interstitial diffusion more effectively than high-collagen tumors [224]. Thus, the upregulation of collagen in the stroma of gliomas makes it more difficult for NT diffusion. In addition, the heterogeneous nature of the collagen networks (and their associated interstitial molecules) further prevents NTs from homogeneously penetrating tumors,

leaving untreated pockets within the tumor mass [225]. A potential strategy to improve NT interstitial diffusion is to degrade the tumor ECM using enzymes such as collagenases [225].

Aside from collagen content, particle-related factors such as electric charge, size and shape also determine the ease with which the NT can traverse the tumor interstitial matrix [226-228]. NTs, which could be hundreds of nanometers in size, have difficulties spreading through the interstitial matrix of the tumor due to limited space [229]. Thus, NT-matrix interactions significantly reduce the amount of nanodrug reaching tumor cells [222]. Nance et al. conducted *in vivo* experiments in which they determined that nanoparticles up to 100 nm in size effectively diffused through normal brain tissue, whereas 200-nm nanoparticles did not [230]. However, it is important to note that the optimal size for deep brain tumor penetration varies across the literature.

12.5 Internalization

A nanodrug might need to traverse hundreds of micrometers of tumor stroma before it reaches malignant cells [231]. Moreover, it is possible that the nanodrug simply diffuses past the cell without recognizing it. Thus, to improve the recognition process, targeting moieties are often conjugated to the NT [15]. For example, we have engineered a dual monoclonal antibody NT for improved drug targeting: the anti-mouse TfR antibody allows the NT to bind the BBB TfR and get transcytosed, whereas the anti-nucleosome antibody 2C5 allows the NT to bind to nucleosome receptors on the cancer cells [232]. It is important to note that the presence of a targeting ligand allows the NT to interact with its respective receptor when within a few nanometers from it; the targeting ligand does not, however, home the NT directly to the receptor from across the tumor mass by using antibodies or peptides [23, 233].

In addition to facilitating cell recognition, the targeting conjugates often promote cellular uptake [234]. Upon binding to their targeted receptors, the NTs are generally shuttled into the cell via endocytosis – the exact endocytic pathway is dependent on factors such as the tumor microenvironment and physicochemical properties of the NT [208]. Yet, receptor-mediated interactions do not always promote internalization [234]. To overcome these non-permitting interactions, other entry mechanisms could be exploited. For example, NTs could be conjugated with cell-penetrating peptides (CPPs) [235]. CPPs are short peptides that are readily internalized by cells, and have been shown to effectively cross the BBB for CNS drug delivery [236]. However, the mechanisms by which CPPs enter cells are poorly understood – they are thought to be taken up through direct translocation, tertiary endocytosis, or other possible mechanisms [208]. An important drawback of CPPs is that they act in a non-specific manner [237], although this problem could be resolved through nanocarriers: in order to overcome their lack of specificity, CPPs can be covalently coupled to target-specific NTs. This covalent coupling allows CPPs to act specifically on targeted cancer cells and shuttle the covalently linked cargo into the target cell [235].

12.6 Endosomal Escape

Currently, endocytosis is the most common route for cellular uptake of NTs: this holds true for both CPP-mediated and receptor-mediated internalization [238, 239]. Regardless of the

precise mechanism of endocytosis, the NT enters the cell encapsulated in an endosome. Aside from caveolae-mediated endocytosis, the downstream fate for all other endosomes is degradation through lysosomal digestion [238]. Thus, to evade lysosomal fusion and digestion, the NT must be able to escape the endosome in a timely manner. Within the literature, there are a variety of mechanisms by which endosomolysis has been achieved. One of the most common mechanisms is via the action of polyethyleneimine (PEI), a polymer with many unsaturated amino groups [233]. PEI acts by binding endosomal protons, causing counter ions and water to be pumped into the endosome. The increased osmotic pressure induces swelling, causing the endosome to burst and release its cargo directly into the cytosol [233].

Yet, PEI has been reported to have undesirable cytotoxic effects [240]. In light of this, another common technique for endosomal escape is via the action of CPPs, which are able to induce endosomolysis under sub-toxic concentrations [241]. However, the mechanisms by which they function vary significantly and require extensive mechanistic studies [208, 242]. Nonetheless, pH-sensitive peptide residues possess great potential for overcoming endosomal entrapment, as endosomes become more acidic as they proceed from their early to late stages (Fig. 10, [12]). Thus, other peptide-based formulations have been composed. For example, we have achieved successful endosomolysis within GBM cells through a pH-sensitive tripeptide, trileucine (LLL). LLL is activated by the acidic environment of the late endosome causing disruption of the endosomal membrane to facilitate cargo release [12]. Furthermore, physical manipulations for endosomal escape such as temperature- and photo-induced disruptions have been reported [243].

12.7 Tumor-Suppressing Activity

At the point that NTs escape the endosome, they enter the cytoplasm. In virtue of their size, NTs are able to act within cells on a molecular level [244] and are programmed to act within the cytoplasm. Depending on their action, the NTs can either act on structures within the cytoplasm or on specific organelles.

One preferred mechanism of action in the cytoplasm involves the use of RNA-interfering agents, primarily AON and siRNA. We have synthesized a glioma-targeting nanobioconjugate that suppressed tumor activity through silencing by AON the expression of two chains (α4 and β1) of vascular laminin-411 overexpressed in GBM [12, 39]. Similarly, siRNA can be used to interfere with the translation of tumor-promoting gene product. For example, Kozielski et al. synthesized siRNA-loaded polymeric nanoparticles that targeted GBM cells. When the nanoparticles entered the reducing cytoplasmic environment, the siRNA was cleaved off their platforms and could bind to their respective mRNAs [245].

Certain tumor-suppressing mechanisms require access to particular organelles. The current standard of care for gliomas, TMZ, acts through such a mechanism. For example, we synthesized a PMLA-TMZ nanoconjugate, which released TMZ into the cytoplasm of glioma cells upon endosomal escape. TMZ, a DNA-alkylating agent, then had to enter the nucleus by diffusion to exert its effects on DNA [13]. However, certain complexes could be too large to enter the nucleus by diffusion, and must interact with the nuclear pore complex

to gain access to the interior of the nucleus. To accomplish this, larger complexes could be conjugated with nuclear localization sequences, which allow them to specifically bind to the nuclear membrane [157]. Similarly, if a NT target is the mitochondria, it could be actively transported across the mitochondrial membrane via a mitochondrial localization sequence.

13 Future Directions

Although enormous challenges exist in brain tumor treatment, we are confident that nanomedicine is able to confer great advantages to brain drug delivery. One very interesting application for nanomedicine in the treatment of brain cancer is the effective delivery of gene editing tools like CRISPR/Cas9. Although the "off-target" effect is still a big concern, a variety of applications of CRISPR/Cas9 system as therapeutics are currently under investigation [246, 247]. In cancer treatment, one strategy to use this technology is to attack oncogenes or modify the epigenome of the cancer cells by CRISPR/Cas9 [248]. After its first adaption for genome editing in eukaryotic system [249, 250], CRISPR/Cas9 system has been rapidly developed into a powerful research toolset as well as a promising therapeutic approach [251]. However, no effective method for intravenous, targeted delivery of CRISPR/Cas9 and its penetration through the BBB is readily available. Thus, nanomedicine holds a great potential to solve this problem and revolutionize both the fields of gene editing and the treatment of brain cancer [252].

Another area of potential innovation in brain cancer treatment is the focus on multimodal drug combinations. Nanomedicines have the ability to carry different therapeutic agents simultaneously and this ability can confer great advantages in cancer treatment and tumor suppression, if given appropriate targets. Defining the most effective use of covalently conjugated and encapsulated (or both) drugs could take advantage of synergistic, additive, and potentiative models of chemotherapeutic drug delivery [253]. In the meantime, the impressive treatment efficacy of cancer immunotherapies has gained attention from the field of nanomedicine. The simultaneous delivery of molecular inhibitors to modulate tumor microenvironment and IL-2 cytokine as part of anti-HER2 fusion monoclonal antibody to boost tumor immune system at the same time was introduced by engineering a nano conjugate to successfully treat HER2+ breast cancer in an animal model [20]. The development of nano immunotherapy is well underway. A novel nano vaccine recently reported to treat tumors, is composed of a mixture of an antigen and a synthetic polymeric nanoparticle, PC7A NP, which generates a strong cytotoxic T-cell response. The PC7A NP activated type I interferon-stimulated genes (STING), which led to tumor growth inhibition in melanoma, colon cancer, and human papilloma virus-E6/E7 tumor models. The combination of the PC7A nano vaccine and an anti-PD-1 antibody showed great synergy in mice [162, 254]. Additionally, nanoparticles have been used to engineer human stem cells to express an apoptotic inducer, such as TNF-related apoptosis-inducing ligand (TRAIL), for treating GBM in a mouse model [255].

In the field of brain imaging, we clearly need not only an improvement in diagnostic applications, but also an advanced imaging technique to guide treatment and this may be extremely helpful in achieving real precision medicine. The ability to predict recurrence of aggressive brain tumors like GBM would improve physicians' ability to design effective

treatment regimens, and provide new knowledge about cancer cell migration and penetration patterns. For example, a mutation in IDH1/2 in GBM was found to correlate with better survival as compared with tumors with WT-IDH genes, and the mutated IDH genes can be detected by MR imaging [256, 257]. Another study found a correlation between metabolic ratios of certain factors (choline-containing compounds (Cho: N-acetyl aspartate and creatinine) in the recurrence pathway of GBM using H-MRS imaging techniques. Problems with this method still exist, including low spatial resolution and long acquisition time [194, 258]. Nanoimaging agents may therefore be able to provide better resolution. Furthermore, we recently showed that differential diagnosis of brain tumors of different origins (primary vs. metastatic tumors) can be effectively made using MRI [14]. In this regard, using imaging methods to understand and detect cancer invasion, distribution of drugs *in vivo*, and tumorigenesis in real-time may bring new mechanistic discoveries in target identification and management, and lead to a more effective treatment [134, 186]. In the light of modern scientific achievements in cancer immunology and a comprehensive individual genomic/proteomic/metabolomic tumor characterization, the versatile covalent nano drug delivery systems can provide unlimited imaging and treatment possibilities for cancers that are difficult to treat and for many other pathological conditions.

Acknowledgments

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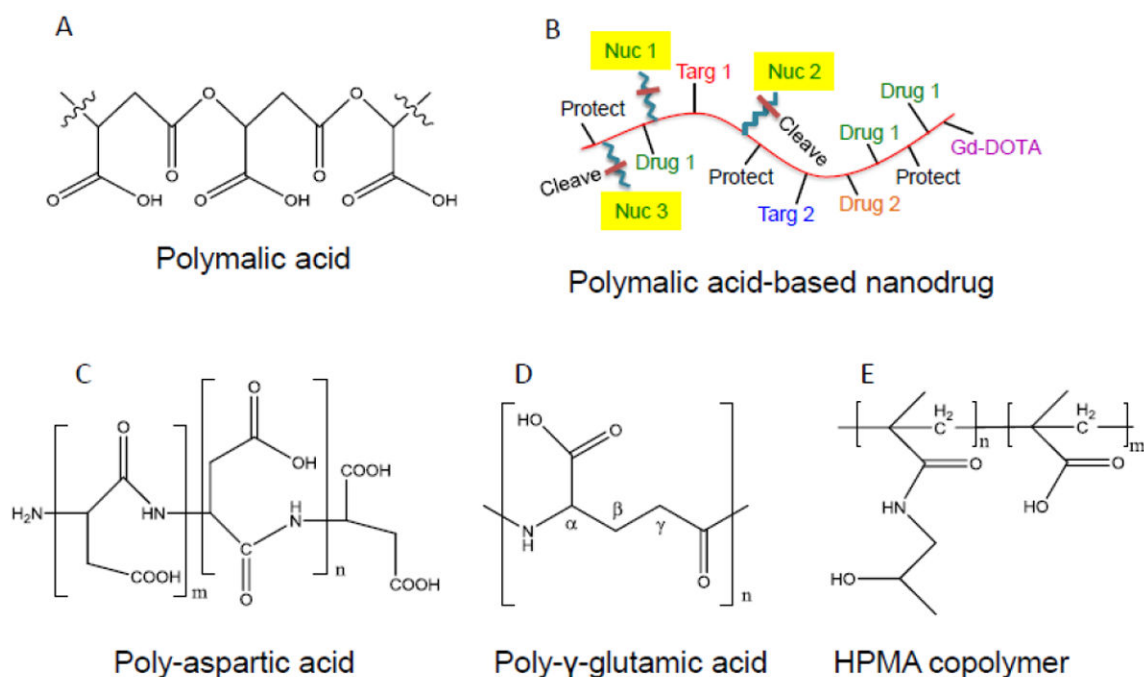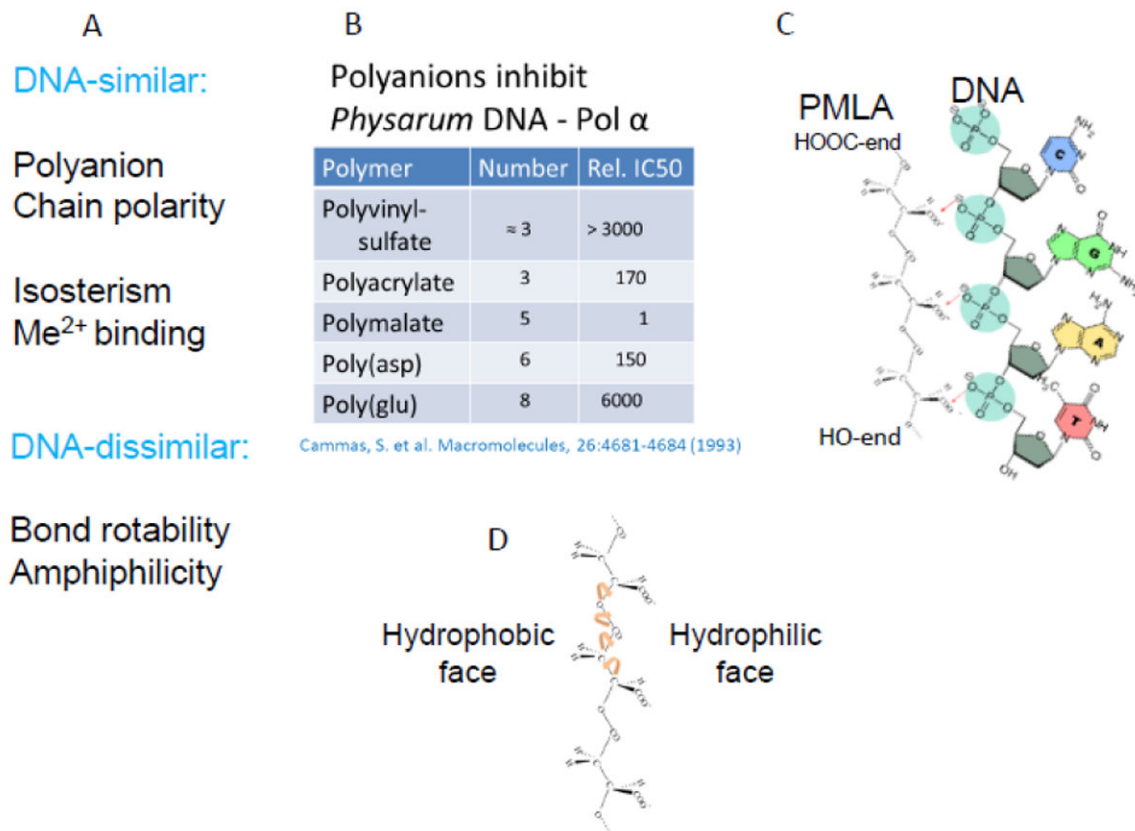
Figure 1.
Common therapeutic polymers for nano imaging and therapeutics.

A, β-Poly(L-malic acid). B, β-Poly(L-malic acid)-based covalent delivery system (CDS). C, α-Poly(L-aspartic acid). D, γ-Poly(L-glutamic acid). E, Poly(N-(2-hydroxypropyl)methacrylamid copolymer). Functional modules are substituted at carboxylic groups. B, an example is shown for the variability of functional modules on a polymeric nanodrug: "Drug 1, Drug 2", multiple copies of different chemotherapeutic drugs; "Nuc 1, Nuc 2, Nuc 3", multiple different AONs; "Targ 1, Targ 2", multiple different targeting antibodies or affine peptides; "Gd-DOTA", Gadolinium-DOTA, an MRI tracer for imaging; "Protect", polyethylene glycol PEG2000 or PEG5000; "Cleave", linker cleavage site.
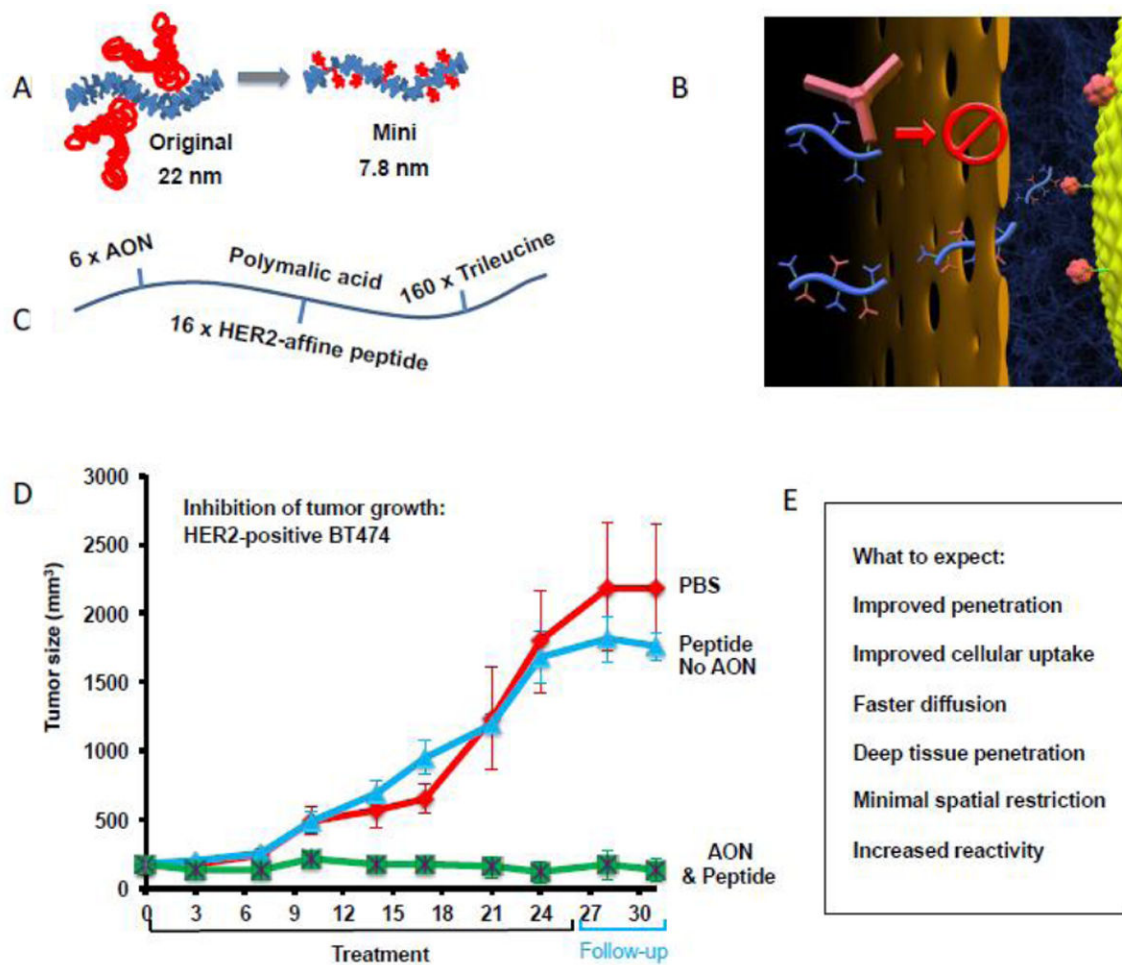
Figure 2.
Concept of a mini nano drug. A, comparison of an antibody-targeted covalent drug delivery system with a peptide-targeted mini nano drug. B, cartoon displaying advantages of the mini nano drug over the parent nano drug in gaining access through size limiting passages and crowded extracellular matrix to reach a receptor for binding. C, example of modules built in a mini nano drug carrying AONs for blocking mRNA for HER2 synthesis. Platform (curved line): PMLA, molecular weight 50 kDa. Loading: 6 molecules AON; 16 molecules HER2 affine peptide; 160 molecules trileucine for mini nano drug release from endosome into cytoplasm. D, growth inhibition of HER2-positive human breast tumor in nude mice by the mini nano drug treatment. E, benefits expected from the mini nano drug vs. antibody containing nano drug. Modified from [23].

A

DNA-similar:

Polyanion
Chain polarity

Isosterism
Me^{2+} binding

DNA-dissimilar:

Bond rotability
Amphiphilicity

B

Polyanions inhibit
Physarum DNA - Pol α

Polymer	Number	Rel. IC50
Polyvinyl-sulfate	≈ 3	> 3000
Polyacrylate	3	170
Polymalate	5	1
Poly(asp)	6	150
Poly(glu)	8	6000

Cammas, S. et al. Macromolecules, 26:4681-4684 (1993)

C

PMLA HOOC-end   DNA

HO-end

D

Hydrophobic face   Hydrophilic face

Figure 3.

Diversity of macromolecular delivery systems distinguished by number, structures and functions of modules. Summary of structural and functional properties of PMLA from *Physarum polycephalum* and comparison with properties of nucleic acids: A, properties of PMLA and nucleic acids as similar and dissimilar. B, competitive inhibition of *Physarum* DNA polymerase-α comparing PMLA and other polyanions. The degree of inhibition correlates with the distance between negative charges. C, comparison of charge distances in DNA and PMLA. D, evidence of PMLA amphiphilic structure revealed by positioning of hydrophobic aliphatic groups and hydrophilic carboxylates.
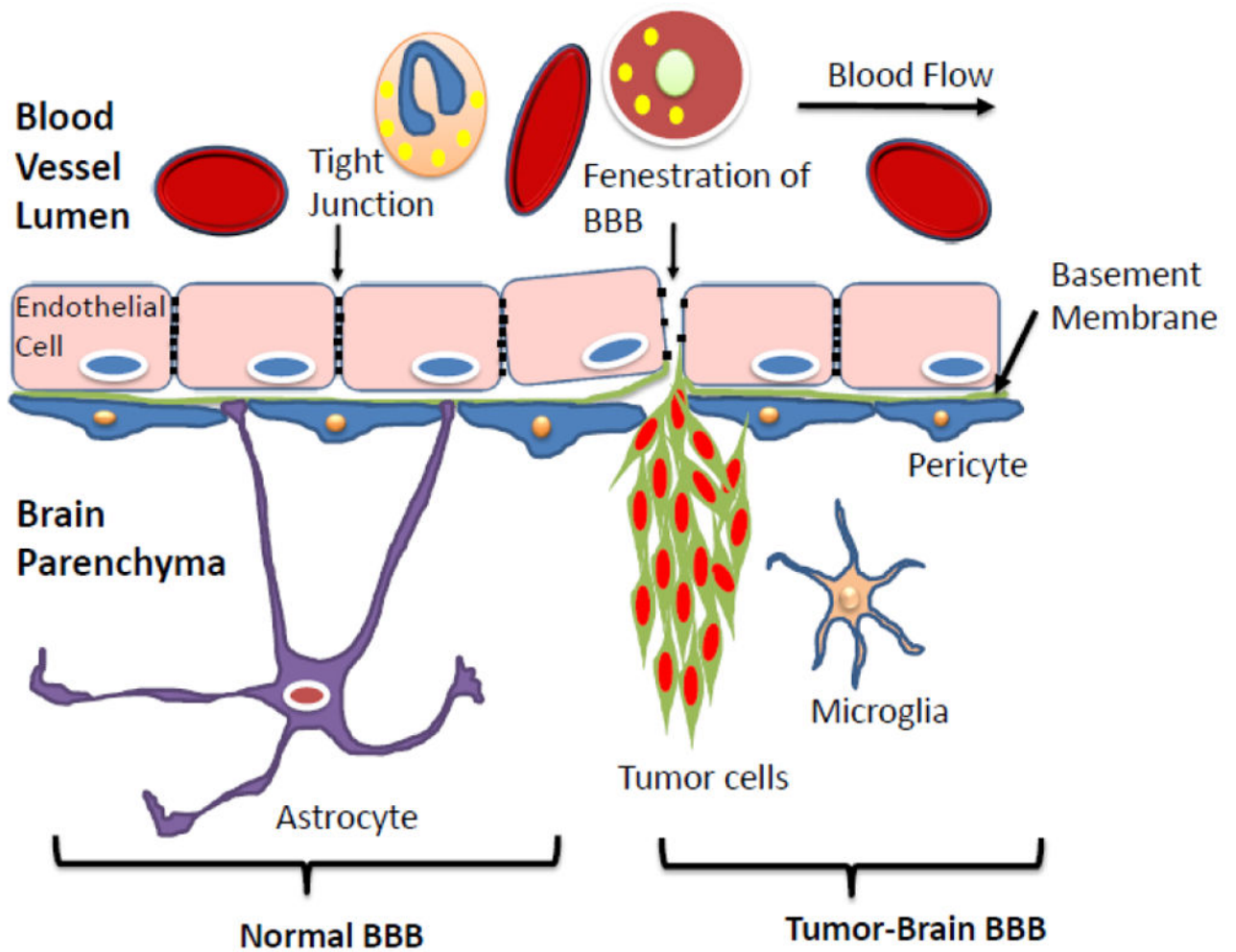
Figure 4.
Blood-Brain-Barrier (BBB) architecture in normal and tumor brain.
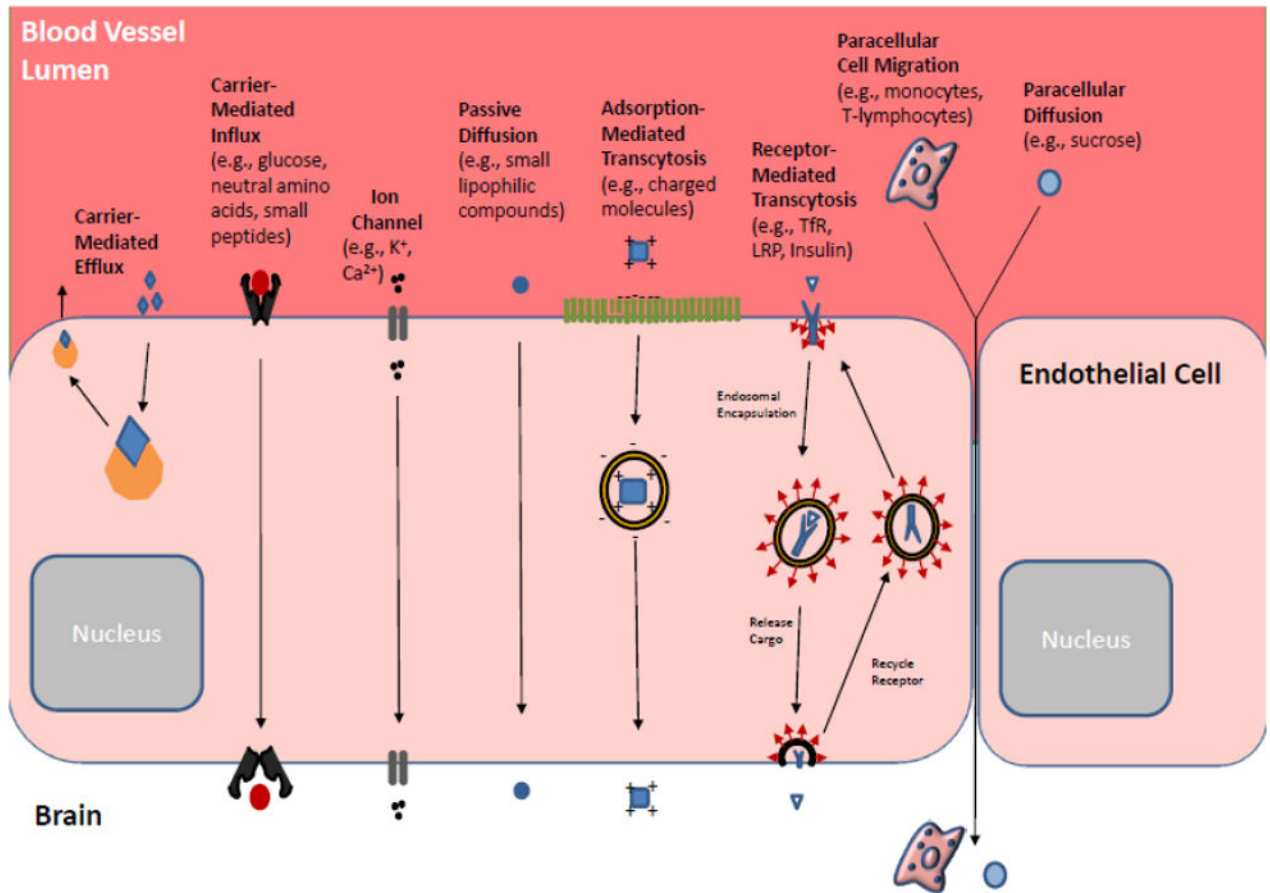
Figure 5.
Molecular transport across normal BBB. In the healthy BBB, there are many mechanisms by which endogenous substances are able to migrate from blood to brain. In addition to simple diffusion, entry mechanisms involve channels, carriers, and receptors.
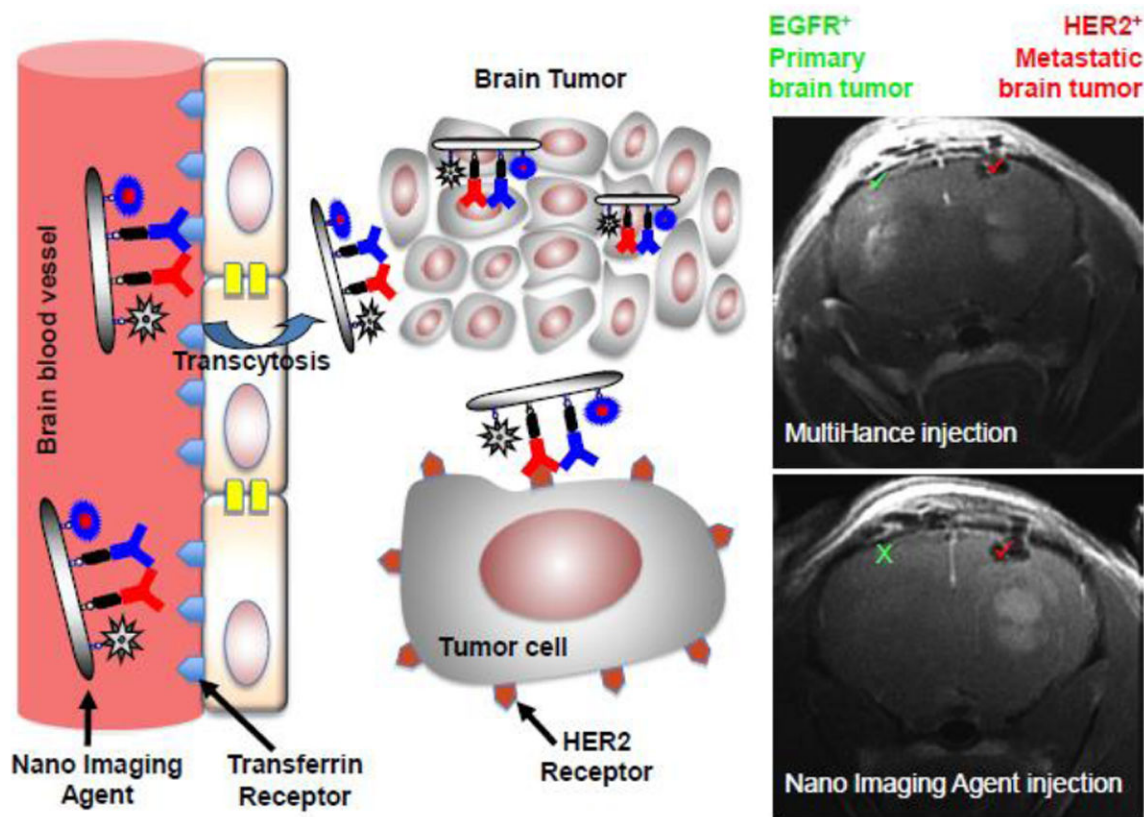
Figure 6.
Schematic illustration of differential diagnosis of brain tumors by MRI using systemically administered Gd-conjugated polymeric nano imaging agents. Mice had different tumors grown in each hemisphere, an EGFR+ glioblastoma and HER2+ breast cancer. Using BBB-permeating and HER2-targeted PMLA-based nano imaging agent it is possible to noninvasively diagnose by MRI the HER2+ tumor (it is the only one visible), which can be subsequently treated with a similar nano agent (see Fig. 8). This is not possible with a standard MRI agent MultiHance that highlights both tumors. Reproduced from [14].

FUS or Hyperthermia
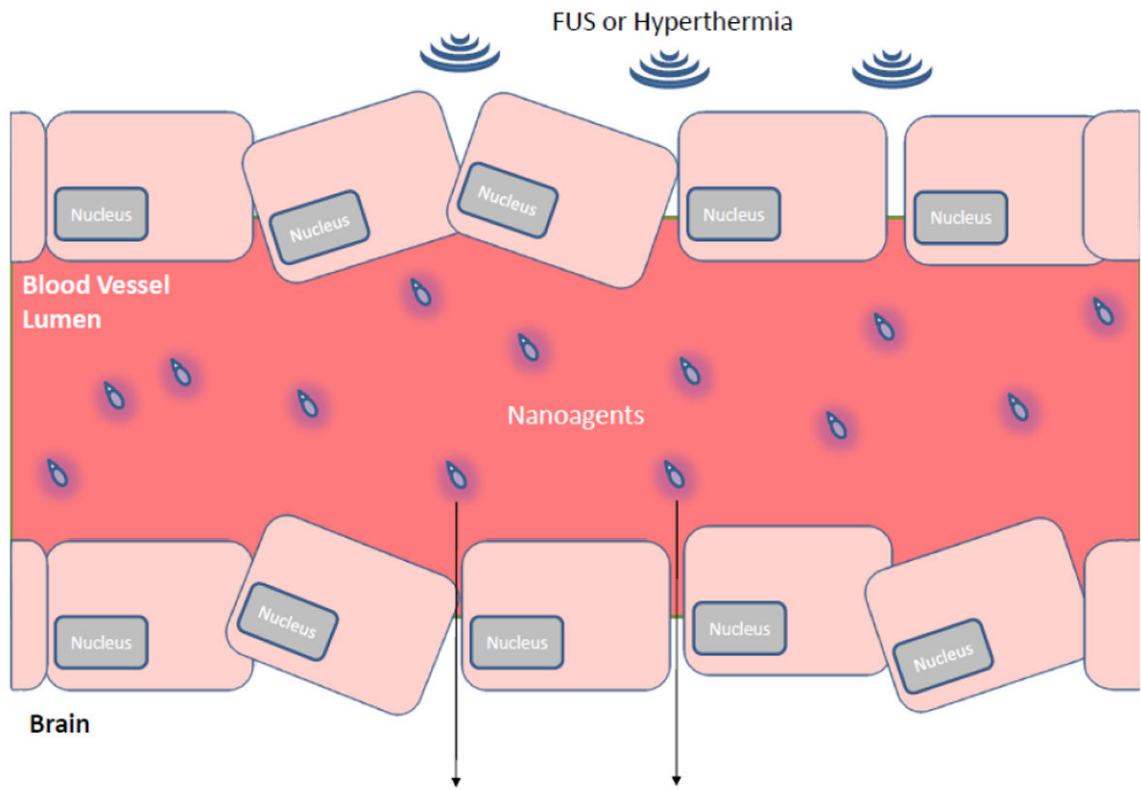
Figure 7.
BBB disruption by physical forces. The two most common physical manipulations to promote CNS drug delivery are shown: Focused Ultrasound (FUS) and Hyperthermia.
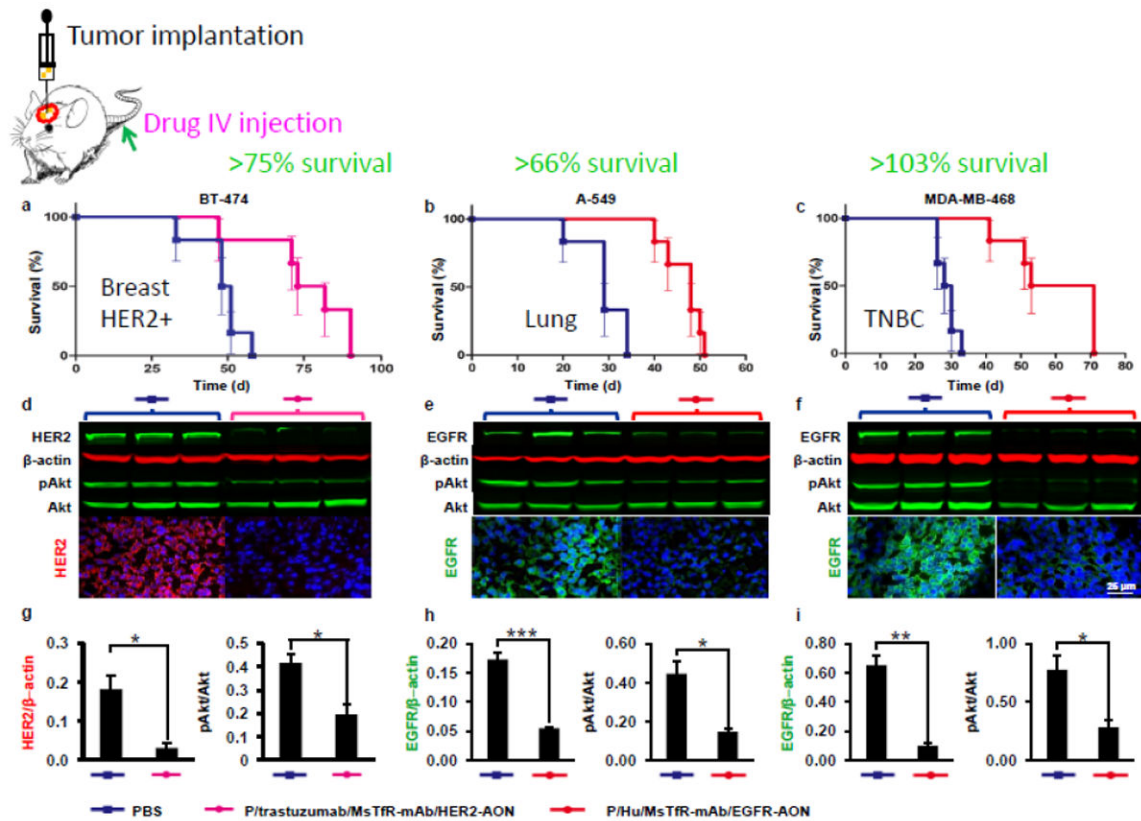
Figure 8.
Survival of mice with brain metastases from primary HER2+ breast, triple negative breast cancer (TNBC) and lung cancers, which are positive for EGFR+, after treatment with tumor-targeted therapeutic polymers inhibiting the expression of HER2 or EGFR. Treatment was performed after MRI-based differential diagnoses of brain lesions made with nanoimaging agents (see Fig. 6). Reproduced from [14].
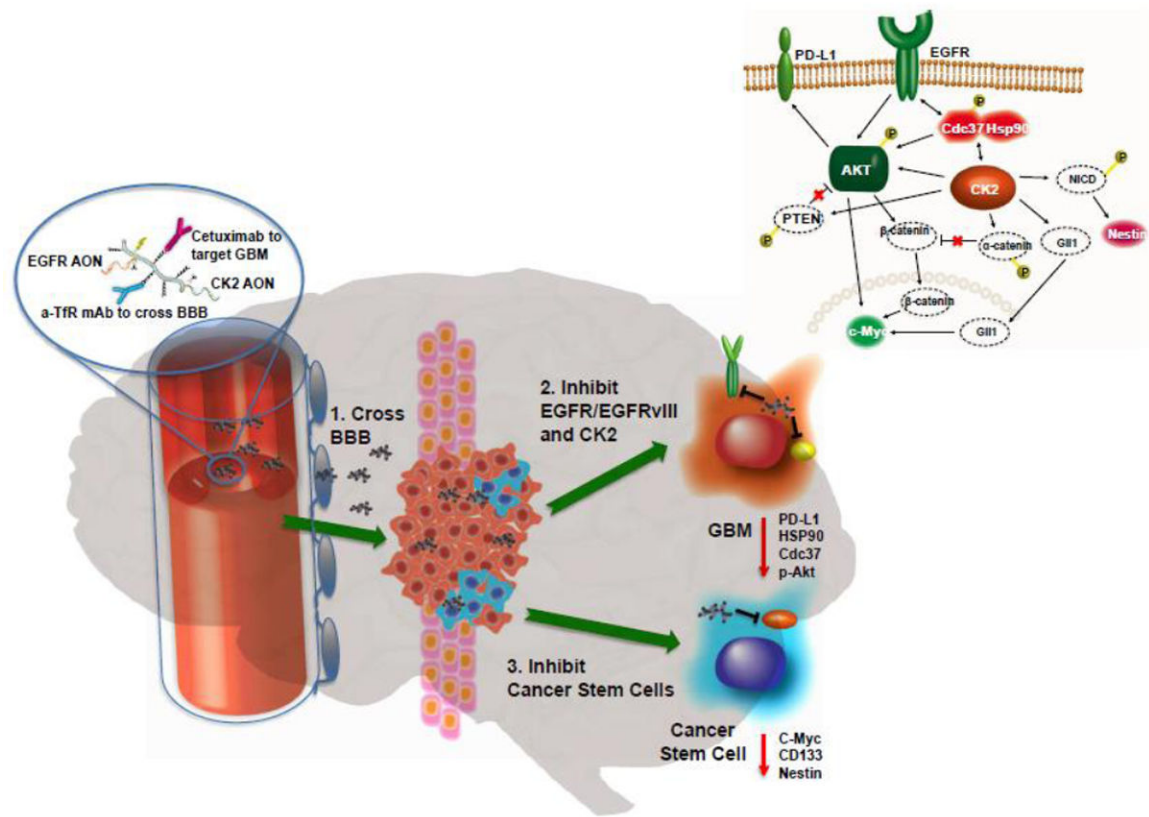
Figure 9.
Schematic illustration of the inhibition of brain tumor growth and glioma stem cells by PMLA-based nano drugs. A novel mechanism of cross-talk between protein kinase CK2, an ubiquitous serine/threonine protein kinase, and wild and mutated variant vIII (EGFR/EGFRvIII) EGFR pathways is depicted. Reproduced from [198].
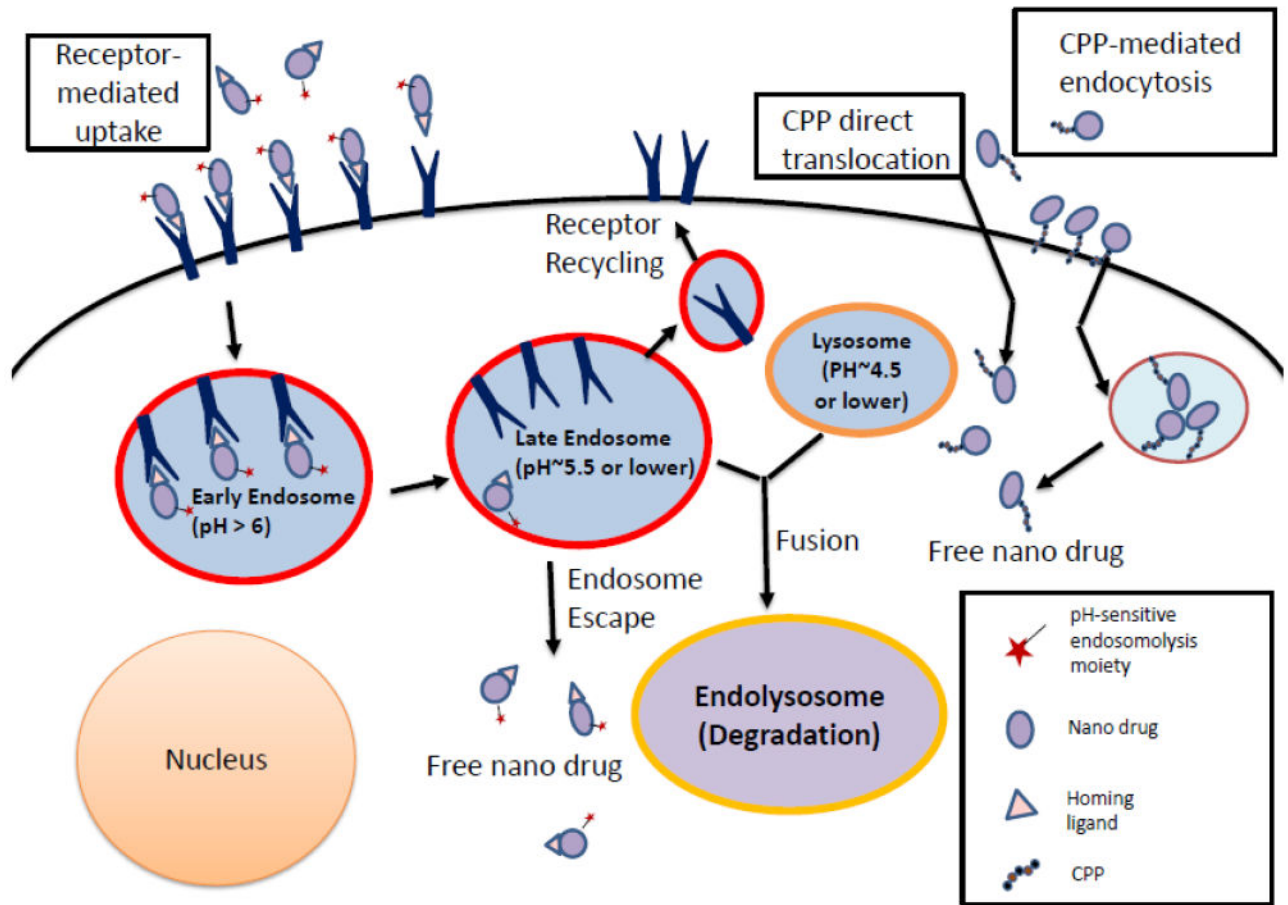
Figure 10.
Receptor-mediated uptake and cytoplasmic trafficking of polymeric nano drugs.
Modifications of nano drugs with homing ligand (antibody or peptide) or with cell
penetrating peptide (CPP) are shown.

Table 1

Properties of therapeutic polymers

Therapeutic Polymer

Dual bonding
Target & Drug (Prodrug)

Multiple bonding
by branching:
Dendrimer

Copolymer suprastructures
containing encapsulated
free drug
Micelle
Solvent enforced micelle
Polymerosomes

Multiple Bonding
Covalent Nano Delivery System with
functional modules for:
Drug (Prodrug Delivery)
Drug release from platform
Protectors against cleavage and systemic
clearance
Imaging agents
Receptor targeting and membrane
penetration:
a. vascular barrier
b. Blood-Brain-Barrier
c. Cell membrane
d. Endosomal membrane
e. Organelle membrane

Table 2

Replacement of antibody by affinity peptide: pro and contra

Pro	Contra
• Multiple peptides per conjugate.	
• Low affinity receptor binding • Does not stick to receptor • Fast release from receptor after internalization	• Less affinity to bind to targeted receptor (HER2)
• Robust structure	• Absence of Fc and lack of biological activity
• Small size and slender shape of the polymeric scaffold	• Small size disfavors passive targeting (EPR)
• Reduced immunogenicity. • Humanization not required	• Limited solubility
• Possibility of multivalency	• Tendency for aggregation
• Easy chemical fabrication • Storage as freeze dried powder	
• Easy storage and clinical handling	

Table 3

Major structures of nanodrugs: advantages and disadvantages for brain delivery

Nanostructure	Advantages for Brain	Disadvantages for Brain
		Comparison with nano covalent conjugates
Liposome ➤Vesicles on one or more phospholipid bilayer with aqueous core ➤50 nm – 5 µm	➤Can cross BBTB through interendothelial gaps ➤Can be functionalized for active transport across BBB ➤Targeted liposomes are shown to accumulate in tumor tissue over normal brain (reduced non-specific toxicity) ➤Can carry both hydrophilic and hydrophobic drugs ➤Inherently non-toxic structure ➤Simple to manufacture [5] ➤Can control physiochemical properties of size, surface charge, and functionalization [6]	➤Low stability ➤Poor reproducibility ➤Low drug loading capacity for insoluble drugs ➤Limited control over drug release ➤Difficult to sterilize ➤Immunogenicity [6] ➤Low circulation time [7]
Dendrimers ➤Highly branched structure, extending from central core ➤Branches are synthetic polymeric macromolecules ➤10-100 nm [6]	➤Uniform size and shape for BBB crossing [6, 7] ➤Many locations for drug/ligand attachment ➤High drug loading ➤High stability [6] ➤Carry both hydrophilic and hydrophobic drugs	➤Complicated synthesis and formulation development ➤Toxicity with amino functional groups [6] ➤Core is difficult to access ➤Some rely on limited diffusion mechanisms, not ideal for BBB crossing [7]
Polymer-conjugates ➤Macromolecular structure, often linear ➤2-25 nm [8]	➤Inherently water soluble ➤Versatility with many regions for moiety attachment ➤Great potential for combinatorial therapeutics ➤Easily functionalized and high drug loading capacity [9] ➤Interactions with self form stable structure [7] ➤Covalent bonds might be present for 100%	➤Conjugate complexity requires careful physical-chemical characterization [9] ➤Potential uncertainty with composition
Micelles ➤Consist of biodegradable and biocompatible amphiphilic block polymers ➤10-100 nm [6]	➤Can carry both hydrophilic and hydrophobic drugs ➤Hydrophilic shell provides stability and long circulation time ➤Small size allows for escape of RES ➤Easy sterilization ➤Popular in siRNA delivery, useful in brain tumor treatment ➤Versatile by polymer block selection	➤Low stability and tend to dissociate ➤Potential premature drug release (particularly bad for brain delivery) ➤Immunogenicity ➤Formulation scale up methods not yet available [6]
Carbon Nanotubes ➤Tube formed of graphene sheet, either open or capped ➤Diameter: 1 nm, length: several micrometers	➤Can penetrate BBB like needle ➤Can be functionalized with moieties covalently or non-covalently [6]	➤Not inherently soluble ➤Generate toxicity and biosafety issues ➤Difficult for targetable moieties to be attached

Nanostructure	Advantages for Brain	Disadvantages for Brain
		➤Low reproducibility [6]
Gold Nanoparticles ➤Silica core coated with thin gold layer [10] ➤1-200 nm [8]	➤Small enough to pass through brain microvasculature ➤Neutral surface charge, low toxicity, and tissue reactivity for BBB crossing ➤Can uniquely produce heat for therapeutic effect ➤Large surface for functionalization	➤Potential toxicity and immunogenicity ➤Unknown effect of functionalization on biodistribution
Magnetic Nanoparticles ➤Iron oxide core coated with material such as polysaccharide, polymer, lipid, or protein ➤1-200 nm [8]	➤Applications in both brain tumor treatment and imaging ➤Can cross BBB favorably ➤Can be administered systemically and targeted by external magnetic field ➤Demonstrated uptake by malignant brain tumor cells	➤Aggregation and instability ➤Non-specific uptake by RES ➤Potential toxicity and immunogenicity

Table 4

Commonly used imaging technologies for brain cancer management

Imaging Technology	Magnetic Resonance Imaging (MRI)	Computed Tomography (CT)	Positron Emission Tomography (PET)	Ultrasound (US)
Mechanism	Main diagnostic approach for brain cancers. MRI employs a strong magnetic field to force protons of water molecules in the body to align with the field, and the scanner can detect the changes in the direction and rotational axis of protons and the energy released.	CT uses X-ray to generate detailed scans of the areas in the body.	PET uses radiotracer to assess the functions of the tissue or organ, such as blood flow, oxygen or glucose consumption.	Ultrasound utilizes high frequency (> 20 kHz) sound waves to produce biological images and the image is produced based on the reflection of the waves off the body structures.
Strength	Fast, noninvasive, nonradioactive and good spatial resolution.	Fast (minutes ~ half an hour); noninvasive; images can be visualized in 2-D (slice) or 3-D fashion.	Often be used in combination with CT to provide accurate anatomic information.	Non-radioactive and quite versatile; for instance, focused ultrasound (FUS) has been adapted for BBB disruption for drug delivery, treatment of neurodegenerative and cerebrovascular diseases, and neurosurgical small volume tumor ablation [32, 141].
Limitation	Contrast enhancing agent, such as gadolinium has high renal toxicity.	Radiation from X-ray; lack of anatomic information.	Radioactive tracers.	Limited spatial resolution.