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Potential advancements in the treatment of difficult-to-treat glioblastoma through nanoparticle drug delivery

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Nanoparticle-mediated therapeutic compounds delivery to glioblastoma

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Abstract

Introduction: Glioblastoma multiforme is the most common and the most aggressive primary brain tumor, with a median survival of 14 months. This dismal prognostic has turned research towards nanomedicine as a new therapeutic approach that can deliver therapeutic compounds to GBM.

Areas covered: The review covers recent advances in targeted delivery of therapeutic compounds to glioblastoma tumors. To reach the tumors, nanocarriers and their cargo should cross the Blood-Brain Barrier (BBB) standing between the blood stream and the tumor. For that purpose, different peptides to facilitate BBB crossing have been added to the nanoparticles. As result, an increase in BBB crossing was observed. Other significant effort has been devoted to selectively target direct the nanocarrier and its cargo to GBM tumors. Once again, targeting peptides have been used.

Expert opinion: Besides significant advances, a more successful design of nanocarriers for efficient BBB crossing and delivery of diagnostic and/or therapeutic molecules to CNS will be needed to achieve efficient nanomedicine-based therapeutics for glioblastoma. This will require a significant effort improving chemical architecture of nanocarriers, identifying the critical design parameters that might play a key role facilitating both BBB crossing and GBM selective targeting.

Keywords: glioblastoma, nanoparticle, targeting peptide, Blood-Brain-Barrier, cancer, siRNA, miRNA

Article highlights

- Nanomedicine-based therapies are being actively explored to find an effective therapy against glioblastoma due to the bad prognostic of this disease. The main objectives consist in facilitating BBB crossing and in selective target to the tumor or its environment.
- Brain-Blood Barrier represents a hurdle for nanoparticles to enter the Central Nervous System, and so to access, together with its cargo, to GBM. To overcome BBB selective targeting achieved by decorating the nanoparticles with different ligands for receptors and transport systems that facilitate BBB crossing has been widely used.
- The second main objective consists in selectively directing the therapeutic nanocarrier and its therapeutic cargo to tumoral GBM cells by decorating the nanoparticles with ligands for overexpressed proteins n GBM cell surface. A variant of this approach is to direct the nanoparticle to the tumor microenvironment using ligands for certain components of the extracellular matrix.
- Different therapeutic cargos have been used in addition to antitumoral small drugs including miRNAs, siRNAs, enzymatic inhibitors as well as other approaches based on physics-based techniques such as hyperthermia, photodynamics, photodermal therapy , and sonodynamics.
- More knowledge is needed on cellular and molecular biology of the tumor to identify the more appropriate therapeutic targets and on the molecular determinants ruling both nanoparticle BBB crossing and selective targeting and cargo delivery to GBM.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and the most aggressive primary brain tumor, with a very poor prognosis for patients and a median survival of 14 months. The standard treatment is based on surgical resection followed by radiotherapy plus concomitant chemotherapy with temozolomide (TMZ), an alkylating cytotoxic drug whose mechanism of action involves methylation of DNA leading to tumor cell G2/M phase arrest and autophagy. However, inter- and intra-tumor heterogeneity, angiogenesis and infiltration in the surrounding tissues make complete resection by surgery near impossible. Most of chemotherapeutic drugs find difficulties in reaching GBM cells due to the low penetration capacity in the blood brain barrier (BBB) and the blood brain tumor barrier (BBTB), making relapses common which leads to a poor prognosis after diagnosis, so new therapeutic strategies for GBM need to be developed.

Some of the most important deregulated or altered pathways and receptors affecting activity of TMZ and other drugs are involved in tumor progression, proliferation, cell differentiation, cell growth, metabolism and survival: tyrosine kinase receptors such as insulin growth factor 1 receptor (IGF1R), endothelial growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), hepatocyte growth factor receptor (HGFR), phosphatidylinositol-3-kinase/serine-threonine kinase/ mammalian target of rampamycin (PI3K/AKT/mTOR) pathway, mitogen-activated pathways (RAS/MAPK); apoptosis pathways; mutated proteins such as galectin-1, p53 (tumor suppressor protein), murine double minute 2 (Mdm2, oncogenic protein), phosphatase and tensin homologue (PTEN, a tumor suppressor gene), isocytrate dehydrogenase (IDH-1, oncogenic protein); and cell cycle checkpoint pathway [1].

2. Nanoparticles as carriers for therapeutic compounds

Due to the limitations of the above indicated therapeutic strategies, nanomedicine has emerged as a promising tool for GBM treatment. Nanocarriers are versatile platforms that can incorporate ligands on its surface with different purposes: increasing bioavailability, targeting specific receptors, decreasing immunogenicity, controlling the

release of the load in space and/or time, etc. They can be designed to reach GBM cells by passive or active targeting and have no toxicity towards healthy cells. Nanocarriers are also able to encapsulate a cargo (chemotherapeutic drugs, therapeutic biomolecules or molecules for diagnosis), protect it from degradation and deliver it to specific sites of action through targeted delivery.

3. Blood-brain barrier (BBB) crossing in glioblastoma xenografts

The first barrier that a nanoparticle must overcome to reach its GBM target from the blood stream is BBB (Figure 1). The presence of BBB and blood-brain tumor barrier (BBTB) are the major limitations for drug delivery for GBM by either oral or parenteral administration, since its presence prevents the passage of foreign substances from the blood stream [2,3]. There are different transport pathways across the BBB that allow the passage of different substances and drugs into the brain such as: passive diffusion for small lipidic molecules; carrier-mediated transporters that convey brain uptake of certain substrates such as glucose, aminoacids and nucleosides; receptor-mediated transcytosis (RMT) for uptake of macromolecules bound to their selective receptors such as transferrin, insulin, lipoprotein, epidermal growth factor; and adsorptive-mediated transcytosis (AMT) for protein uptake. (Figure 2). Several recent reviews have covered the transport mechanisms used by nanoparticles to cross BBB [4,5] Besides the transport mechanisms to facilitate BBB crossing, the barrier has also efflux pumps for drug extrusion from the brain, being a main obstacle for many drugs, including anti-cancer drugs, to reach therapeutic concentrations. The best known of these extrusion systems are: the p-glycoprotein, ATP binding cassette (ABC) transporter and multidrug resistant protein (MRP). The BBTB exhibits higher permeability than BBB due to pathological disruptions of the BBB caused by the tumor. However, this enhanced permeability does not ensure optimal transport of drugs into the tumor. To overcome the problem of low BBB permeability and to reach glioblastoma in the brain parenchyma, several strategies have been devised (Table 1).

3.1 Invasive strategies

The first group of strategies is based on using drug administration routes different from oral or parenteral ways. These strategies can be invasive, through direct injection into the brain (intracerebral, intraventricular, and intrathecal injections), that is complex to

use for long treatments and can cause significant side-effects including infections, or non-invasive (intranasal route), having limitations in the total volume of drug to be administered, reproducibility and enzymatic degradation of drugs in the nasal cavity. There are two relevant

3.2 Parenteral administration

The most widely used way for facilitating BBB crossing of therapeutic nanocompounds consists in decorating the surface of the carriers/nanoparticles with targeting ligands for achieving RMT or AMT to cross the BBB (Figure 3). Different ligands have been used to decorate nanoparticles to improve BBB crossing such as:

a. PEGylated liposomes decorated with the cell penetrating peptide (CPP) CB5005 (KLKLALALALAVQRKRQKLMPC). This nanosystem, designed for BBB penetration, tumor accumulation and inhibition of Nuclear Factor kB (NF-kB)) was able to penetrate into glioma cells and deliver doxorrubicin into the nucleus and to accumulate into intracranial tumor GBM xenografts prolonging the animals survival time [6].

b. Lipoprotein-receptor-related protein-1 (LRP-1) targeted vesicles. The low-density lipoprotein receptor related protein 1 (LRP1) is a large endocytic receptor also expressed in BBB and glioblastoma cells. Angiopep-2 is a peptide that targets the LRP1 on the BBB with higher brain penetration capability than other proteins, such as transferrin, lactoferrin and aprotinin Protein toxins chaperoned by LRP-1-targeted vesicles, using the peptide Angiopep-2, have emerged as a novel and highly promising modality for glioblastoma treatment [7]. In fact, ANG1005, an angiopep-paclitaxel conjugate has entered clinical trials for treating primary GBM [8, 9].

c. Transferrin (Tf)-decorated liposomes. Transferrin is a serum glycoprotein that transfer iron into growing cells through the transferrin receptor (TfR). This receptor is usually overexpressed in cancer cells. Targeting the TfR may be achieved using the natural ligand, transferrin, or specific peptides, monoclonal antibodies and single chain antibody fragments to the TfR [10].

d. Mannose-liposome-curcumin. Glucose transporter 1 (GLU1), which carries glucose and other monosaccharides through the BBB, has been also used for facilitating BBB crossing. Liposomes composed by phosphatidylcholine, cholesterol, polyethylene glycol and distearoylphosphosphatidylethanolamine were modified with *p*-aminophenyl-α-D-mannopyranoside, a mannose analog. These modified

liposomes did not only promote penetration through the BBB into the brain, but also targeted selected intracerebral regions, including cortex, cerebellum, brainstem, hippocampus, and pontine nuclei [11].

e. . Biocompatible and intracellular pH-responsive short tripeptide (Lys-Phe-Gly, KFG) was used to target doxorubicin-loaded gold NPs to the tyrosine kinase nerve growth factor (NGF) receptors, showing a significantly decrease in cell proliferation and tumor growth in a BT-474 cell xenograft model in nude mice [12].

3.3 BBB transient opening

Another strategy uses focused ultrasounds (FUS) at low acoustic pressures, in the presence of circulating microbubbles (MBs), to open the blood–brain barrier in a non-invasive and reversible way. This strategy was used for the treatment of nude mice bearing intracranial glioblastoma xenograft [13]. Moreover, MBs combined with low-intensity pulsed FUS were also used to transiently open the blood-brain barrier in mice bearing temozolomide-resistant gliomas and to deliver liposomes loaded with the DNA-repairing enzyme O6-methylguanine–DNA methyltransferase (MGMT) inactivator (O6-(4-bromothenyl)guanine). The liposomes targeted MGMT, thereby sensitizing murine and human glioma cells to temozolomide, reduced tumor growth and significantly prolonged survival of glioma-bearing mice, when combined with temozolomide chemotherapy [14].

4. Glioblastoma targeting

Increasing nanoparticle-mediated delivery of therapeutic cargos to GBM cells represents a very promising strategy to approach GBM therapy. Targeting can be done by either passive or active targeting.

4.1 Passive targeting

Drug delivery by therapeutic nanoparticles can be prompted by the enhanced permeability and retention effect (EPR) [15] which is based on structural vascular abnormalities (fenestrations, irregular branching, irregular perfusion) resulting in tumor vasculature leakiness that allows the accumulation of large particles (proteins, macromolecules, micelles, and nanoparticles) into the interstitial space of the tumor at higher levels that in normal tissues [15]. This pathological disorganization of vessels is reinforced by an impaired lymphatic drainage in the tumor microenvironment. The

EPR effect allows larger accumulation of certain molecules in tumors than in healthy tissues increasing therapeutic compounds concentration in the tumoral tissue. However, most nanocarriers deliver their cargo to the tumor by active targeting.

4.2 Active targeting

These strategies are designed for targeting either the tumor microenvironment (hypoxic regions, angiogenesis, fibrin deposition) or, directly, GBM cells through overexpressed cell receptors (Figure 2). Several ligands have been incorporated into the nanoparticles for this purpose (Table 2).

4.2.1 Ligands for GBM cells overexpressed proteins

Several ligands for proteins that are more abundant in GBM tumor cells than in healthy tissue have been used to decorate different nanoparticle types. Among these ligands, we can find:

a. Several ligands for Neuropilin-1 (NRP-1) receptor, over-expressed in tumor cells and tumor blood vessels, have been incorporated into nanoparticles such as: RGERPPR peptide in PEGylated poly(L-γ-glutamylglutamine) nanoparticles [16]; tLyp-1 in niosomes (nanoparticles with a bilayer structure obtained by self-association of non-ionic surfactants and cholesterol in water) [17]; DA7R, a glioma-homing heptapeptide (DRDPDPDLDWDTDA) that also binds to the VEGFR-2 receptor [18]; and the iNGR (CRNGRGPDC), peptide which is composed of a vascular homing motif, a tissue penetration motif (R/KXXR/K), and a protease recognition site which produces the peptide truncated form iNGRt which can penetrate deeper into GBM tumor and bind to its receptor NRP-1 [19]. The peptide FHK (FHKHKSPALSPV) which targets tenascin C has been also used, in conjunction with tLyP-1 to target PEG-PLA polymers to intracranial U87 xenografts in mice [20]. The octameric homing peptide, PL3, that interacts with the C-isoform of Tenascin-C (TNC-C) and with the receptor neuropilin-1 (NRP-1), has been used to decorate iron oxide nanoworms (NWs) and metallic silver nanoparticles loaded with the proapoptotic D(KLAKLAK)2 peptide. The PL3-coated nanoparticles were found to accumulate in TNC-C and NRP-1-positive areas in clinical tumor samples, suggesting a translational relevance.[21]

c. Ephrin type-A receptor 3 (EPHA3) is a membrane-associated receptor which is overexpressed in the stroma and vasculature of gliomas but not in normal tissues. An anti-EPHA3 recombinant, nonfucosylated IgG1j (human f-allotype) monoclonal antibody that targets the EPHA3 receptor tyrosine kinase has entered a phase I clinical trial (KB004) [22].
d. Lysophosphatidic acid (LPA). Loss of primary cilia is related to cell proliferation and has been observed in GBM cells. Lysophosphatidic acid receptor 1 (LPAR1) is accumulated in primary cilia and its signaling through Gα12/Gαq is involved in cancer cell proliferation[23]. Ki16425 is an inhibitor of LPA signaling that has been delivered to brain PEG-PLGA nanoparticles to inhibit tumor progression in vivo.
e. CD133-targeted antibodies. CD133, also known as prominin-1 (PROM-1 or AC133), is a transmembrane glycoprotein family member which has been recently identified in GBM and leukemia cancer stem cells. This selective CD133 location has been used to deliver temozolomide to GBM xenografts using immunoliposomes decorated with anti CD133 antibodies resulting in reduction in tumor size and an increased in median survival time [24].
f. HER2 receptor, a member of the erbB family of tyrosine kinases, is overexpressed in GBM cells surface. Antibodies against this receptor have been used to target copper- and selenium-loaded nanoparticles to GBM xenografts allowing tumour visualization [25]. Cu and Se nanoparticles were attached to HER2 antibody and poly(ethylene glycol)-poly(ethylenimine) (PEG-PEI) copolymers, since PEI has endosome-escape properties due to its proton sponge capabilities and PEG reduces PEI toxicity and enhances biocompatibility. MR imaging technique using SPIONs *in vivo* showed the effective delivery of nanoparticles to the brain [25].
g. Epidermal growth factor receptor (EGFR) is overexpressed in GBM cells and it is involved in cell proliferation, angiogenesis and metastasis representing a good target for GBM therapy. A new theragnostic nanosystem based on radioiodine-labelled anti-EGFR binding nanoparticles have been constructed to evaluate its efficacy for the treatment of GBM. Nanoparticles composed of bovine serum albumin and polycaprolactone (BSA-PCL) were added to anti-EGF antibodies enhancing the uptake and accumulation of BSA-PCL in xenografts in nude mice inhibiting tumor growth [26]. Moreover, TMZ-loaded dual-functionalized 2-methacryloyloxyethyl phosphorylcholine-based NPs with cMBP, a MET (mesenchymal–epithelial transition factor) targeting peptide and Inherbin3, and an

EGFR targeting peptide which inhibits EGFR phosphorylation were able to attenuate DNA damage repair and enhance TMZ sensitivity through the down-regulation of E2F1 in TMZ resistant cells, leading to a significant repression in tumor growth and a prolonged survival of mice after injection of the NPs [27].

h. Programmed death-ligand 1 (PD-L1) is highly expressed on GBM Tumor-associated myeloid cells (TAMCs). A lipid nanoparticle formulation surface-functionalized with an anti–PD-L1 therapeutic antibody was used to encapsulate dinaciclib, a cyclin-dependent kinase inhibitor. Radiotherapy combined with the nano-immunotherapy led to dramatically extended survival of mice in two glioma models [28].
i. Aptamers are oligonucleotides or peptides that interact with targets by recognizing a specific three dimensional structure [29]. Aptamers have been used to target deliver different nanoparticles. So, poly(lactic-co-glycolic)-block-poly ethylene glycol (PLGA-b-PEG) copolymer loaded with dactolisib (a potent PI3K-mTOR inhibitor) were conjugated with an anti-platelet-derived growth factor receptor (PDGFRβ) aptamer for selective targeting against GBM. Upon intravenous injection in nude mice bearing intracranial U87MG tumors, the nanoconjugates crossed the BBB and accumulated at the tumor site delivering dactolisib and inhibiting mTOR activity [30]. Moreover, a tetrahedral framework nucleic acid (tFNA) nanoparticle loaded with TMZ was modified with GS24, a DNA aptamer that can specially bind to transferrin. The NPs were more effective in killing TMZ-sensitive cells by activated apoptosis and autophagy pathways and overcame the resistance of TMZ resistant cells via consuming MGMT. They were able to cross the BBB of mice [31]. Gold NPs have been also used to encapsulate the aptamer U2 that targets EGFRvIII. The nanosystem was able to inhibit the proliferation and invasion of U87-EGFRvIII cell lines and prolonged the survival time of GBM-bearing mice through the inhibition of the EGFR-related pathway [32].
j. Borneol, a natural terpene, is a competitive inhibitor of P-glycoprotein present in the vascular endothelial cells of the BBB that allows other drugs with lower affinity for P-gp to accumulate in the brain. Taking advantage of these properties, doxorubicin-loaded PEG-nanomicelles were functionalized with borneol which significantly inhibited the tumor growth and metastasis of GBM models in mice [33].
k. Hyaluronic acid can bind to the CD44 receptor, one of the cluster of differentiation (CD) proteins, which is overexpressed in many types of tumors, including GBM. A hybrid nanocarrier system based on hyaluronic acid-modified polymer has been

used to transport docetaxel to GBM cells. HRK-19 peptide (HAVRNGRRGDGGAVPIAQK) is another important part of this multi-targeting hybrid nanocarrier system. It can disrupt cadherin-mediated cell adhesion. The resulting NPs were able to cross the BBB with negligible systemic toxicity and enhanced therapeutic efficacy, exhibiting significantly improved survival rates in intracranial C6 glioma-bearing rats [34].

l. LinTT1. Paclitaxel-loaded iron oxide nanoworms, silver nanoparticles and albumin nanoparticles modified with LinTT1 (AKRGARSTA), a novel tumor penetrating peptide that targets cell surface p32. LinTT1-guided proapoptotic NPs exerted strong anti-glioma activity in two models of GBM, including doubling the lifespan of the mice in an aggressive orthotopic stem cell-like xenograft.[35]

4.2.2 Ligands targeting extracellular matrix

Another possibility for actively targeting glioblastomas relies on the use of extracellular matrix ligands to accumulate therapeutic compounds in the tumor vicinity. Several ligands have been incorporated into nanoparticles to explore this approach.

a. Chlorotoxin (CTX). Matrix metalloproteinase-2 (MMP-2) is an extracellular matrix degrading enzyme, playing an important role in tumor invasion. CTX is a peptide derived from an Egyptian scorpion venom, which has initially been characterized as an MMP-2 inhibitor and also as a voltage-gated chloride channel blocker. CTX exhibited high specificity, selectivity and affinity for GBM, so it has been extensively used as a ligand for active targeting GBM. A combined use of CTX-nanovectors with radiotherapy have been reported as a promising therapeutic towards GBM [36].
b. Fibronectin, which is overexpressed in the near perivascular extracellular space of GBMs, has been targeted through mesoporous silica shell and an iron oxide core modified NPs loaded with 1400W, a potent inhibitor of the inducible nitric oxide synthase (iNOS). This enzyme is preferentially expressed in brain tumor initiating cells (BTICs), a highly plastic cellular subpopulation that is resistant to current therapies. External low-power radiofrequency field triggered rapid drug release and disrupted the BTIC population in hypoxic niches, suppressed tumor growth and significantly increased survival in BTIC-derived GBM xenografts.

c. The tripeptide RGD binds specifically to αvβ3 integrins that are overexpressed on neurovascular endothelial cells. Thus, cyclic RGD peptide-decorated poly(ethylene glycol)-b-poly(e-caprolactone) micelles [37] or PEG-DSPE liposomes [38] increased doxorubicine delivery to GBM xenografts prolonging survival time in nude mice. Paclitaxel-loaded RGD-linked PLGA (poly lactic-co-glycolic acid) NPs administered by intranasal application were able to control the tumor burden (75 % reduction) by inducing apoptosis and/or inhibiting cancer cell proliferation without affecting normal brain cells [39]. Diamino propane tetraiodothyroacetic acid (DAT), is a thyroid hormone derivative that blocks the actions of thyroid hormones at both the T4/T3 receptor site and the T3-specifc receptor site on integrin αvβ3 controlling tumor cell proliferation. RGD dimer (RGD2, Glu-{Cyclo[Arg-Gly-Asp-(D-Phe)-Lys]}2) and lactoferrin have been also used to decorate small-size gadolinium oxide nanoparticles stabilized with poly(acrylic acid) polymers and bovine serum albumin for theragnostics in orthotopic GBM models. The NPs showed high biocompatibility and the ability to cross mice BBB, through lactoferrin receptor-mediated transcytosis due to its small particle size (13.4 nm). The NPs behaved as an effective radio sensitizing agent enhancing radiation therapy effectivity in GBM xenografts [40].

5. Therapeutic approaches

Different therapeutic approaches have been explored in glioblastoma xenografts (Table 3).

5.1 Gene therapy

Gene therapy can be defined in a broad sense as the use of genetic material as a therapeutic tool. Different types of genetic materials can be introduced in different cells ranging from full genes aimed to replace a malfunctioning one (classical use of gene therapy) to small RNA molecules (miRNA or siRNA) (Figure 4) aimed to specifically remove a protein or a specific signaling molecule. In this review we will focused on the use of small RNA molecules.

5.1.1. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small (18-22 nucleotides) non-coding RNAs that regulate gene expression by directly targeting messenger RNAs (mRNAs), resulting in mRNA degradation or translational repression [41]. They play a very important role regulating different cellular mechanisms including cell differentiation and proliferation [42]. Both miRNAs and siRNAs have been vehiculized, using nanoparticles, to GBM cells to interfere with their survival and/or proliferation [43,44].

Therapeutic miRNAs (antimiR-100 and antimiR-21) have been also used to allow presensitization of GBM cells to the systemically delivered chemotherapeutic drug TMZ increasing the survival of cell-derived orthotopic xenograft models in mice after intranasal delivery of NPs. The miRNAs were encapsulated in gold-iron oxide nanoparticles coated with β-cyclodextrin-chitosan hybrid polymer and functionalized with PEG-T7 peptide using CD-adamantane host-guest interactions [45].

5.1.2. Small interfering RNA (siRNA)

siRNAs are exogenous activators of the RNAi system that can degrade homologous mRNAs [46]. They have been widely used to knockdown different proteins involved in GBM biology [47] including multidrug-resistance associated protein 1 (MRP1) whose overexpression in GBM cells is one of the main responsible for chemoresistance towards drugs such as TMZ and vincristine [48]. Thus, gold-containing nanoparticles have shown good profiles to efficiently transfect specific siRNA to decrease the target protein levels and providing the complex with theragnostic properties [49,50]. Moreover, superparamagnetic iron oxide nanoparticles (SPIONPs) targeted to EGFR were used to encapsulate both anti-survivin siRNAs and the anti-cancer drug doxorubicin. In vitro and in vivo studies demonstrated that the EGFR-NPs exhibited excellent targeting specificity towards GBM stem cells (GSC). They improved the therapeutic efficacy by effectively knocking down survivin protein levels and enhanced the sensitivity of GSCs to the anticancer drug doxorubicin. A further step in the use of siRNA consists in employing more than one siRNA aimed to different targets to be transported by nanoparticles. This novel strategy is acquiring more relevance for GBM therapies [51]. Thus, cationic polymeric NPs functionalized with angiopep-2 peptide were used for dual siRNA transfection: co-targeting of polo-like kinase 1 and vascular endothelial growth factor receptor-2 resulted in effective suppression of tumor growth and significantly improved survival time of nude mice bearing orthotopic GBM brain tumors [52]. Moreover, solid lipid NPs (SLNs) targeted with the iRGD peptide

(CCRGDKGPDC) have been also used for the co-delivery of siRNAs against both EGFR and PD-L1. Radiation therapy followed by systemic administration of targeted SLNs leads to a significant decrease in glioblastoma growth and prolonged mouse survival [53].

5.2 Enzyme inhibition

Inhibition of key enzymes involved in GBM proliferation and resistance to therapy is becoming a promising therapeutic approach to GBM. Histone deacetylases (HDACs) are frequently overexpressed in GBM cells. Some HDCAs inhibitors, like quisinostat, have demonstrated a potent in vitro efficacy for cell cycle arrest and apoptosis induction but its clinical application is limited due to its low biodisponibility. Poly(D,L-lactide)-β-methoxy poly(ethylene glycol) nanoparticles (PLA-PEG) were used to encapsulate quisinostat and increase its water solubility. These nanoparticles succeeded to slow orthotopic GL261-induced GBM tumour growth and prolong survival of treated mice in comparation with control untreated animals [54].
Heat shock protein A5 (HSPA5), is a member of HSP70 family. It is significantly upregulated in cancer cells and helps tumor cells to detect and repair irradiation-induced protein and DNA damage. Gold NPs coated with the photothermal conversion agent polydopamine (PDA) for enhanced radiotherapy and photothermal therapy was loaded with Pifithrin-μ (PES), a HSPA5 inhibitor that disrupts the association between the protein and its cofactors. The NPs were able to activate pro-apoptotic unfolded protein response cascades, leading to remarkably improved radiotherapy and photothermal therapy efficiencies [55].

5.3 Hyperthermia

Hyperthermia is a treatment for cancer based on administering heat in the range of 40-46 ºC to trigger cancer cells apoptosis selectively. Magnetic fluid hyperthermia delivers thermal energy through superparamagnetic iron oxide particles (SPION) exposed to an alternating magnetic field. Magnotosomes are membranous structures present in magnetotactic bacteria containing iron magnetic particles covered with a lipid bilayer membrane such as magnetite crystals (ferric and ferrous oxide mineral) [56]. After 68 days of administration of magnetosome nanoparticles covered with poly-L-lysine and following application of hyperthermia (42 ºC, 27 magnetic sessions) to mice bearing intracranial U87 GBM xenograft, the tumor fully disappeared in all the animals [57].

Superparamagnetic iron oxide nanoparticles (SPIONs) coated with silicate mesolayers and carbon shells have preference for cancer cells because of a higher rate of uptake by these cells and a pronounced adherence to cancer cell membrane. Even in an ultralow alternate magnetic field, nanoparticles generated sufficient heat to cause tumor death. Both in vitro and in vivo models of the blood-brain barrier evidence the ability of nanoparticles to cross the barrier [58].

The combination of multi-targeting magnetic nanoprobes has potential to enhance magnetic induction of hyperthermia in the tumor. Magnetic iron NP functionalized with a biocompatible shell of DSPE-PEG_{2000}, RGDyK peptide and glucosamine have been used as a theragnostic agent for targeting neovascular endothelium and tumor cells, following a tumor-suppressive effect through hyperthermia under an alternating current magnetic field [59]. Silica nanoparticles with magnetic core ($ZnFe_2O_4$) functionalized with iRGD peptide moieties together with ROS-generating agent (sodium nitroprusside, SNP) and an ROS-scavenger-inhibitor (diethyldithiocarbamate, DDC) were able to abate selectively tumor cells via magnetic hyperthermia [60].

5.4 Photodynamic, photothermal and sonodynamic therapies

Photodynamic therapy (PDT) uses light to activate non-toxic photosensitizers to generate cytotoxic reactive oxygen species that kill cancer cells, shutdown microvasculature and activate the immune system. This method has been used to inhibit the growth of U87MG xenografts in mice brain [61] and, in conjunction with photothermal therapy, to increase survival of GBM xenografted mice [62]. Combination of photodynamics and chemotherapy has been also reported. A casein nanoformulation with the integrated photosensitizer indocyanine green (ICG) was loaded with genistein. ICG is a hydrophilic tricarbocyanine near-infrared (NIR) emitting (650–850 nm) fluorophore that has been clinically approved by the FDA with high tissue penetration. ICG produces singlet oxygen and heat upon NIR irradiation and is thus exploited as a therapeutic compound to be used in both PDT and PTT. Genistein is an inhibitor of the protein-tyrosine kinase (PTK) which attenuates cancer cell growth by inhibiting PTK-mediated signaling. The NPs triggered oxidative stress, activating the apoptosis cascade, promoting cell cycle arrest and damaging the mitochondrial membrane potential, collectively causing GBM cell death. NPs robustly accumulated in the brain after crossing the BBB with no signals of toxicity in vivo [63].

Photothermal therapy (PTT) is based on the administration and intra tumor accumulation of plasmonic NPs that, after illumination with a light of a certain wavelength, produces synchronized oscillations through band electron conduction to convert near-infrared (NIR) light intro heat (hyperthermia) which kills cancer cells. The main mechanisms of cell death are cell membrane destruction, tumoral denaturation and angiogenesis blocking. Hybrid metal-organic nanomaterials having GSH-responsive activation have demonstrated an enhanced photodynamic therapy efficacy through intracellular MnO_2 reduction [64].

Sonodynamic therapy is based on the use of low-intensity ultrasound (US) irradiation combined with nontoxic sonosensitizers to induce reactive oxygen species (ROS) to get cancer cell death and tumor suppression. It has the advantage of using high-penetration US waves instead of light. In this context, treatment of orthotopic mouse models of GBM with human serum albumin nano assemblies conjugated with N-chlorin e6, a second-generation photosensitizer with antitumor activity when used in conjunction with irradiation, caused a reduction in tumor size and increased animal survival [65]. Moreover, suppression of tumor growth in a C6 tumor xenograft model was achieved via sonodynamic therapy using holo-transferrin (holo-Tf, endogenous Tf with the highest affinity with Tf receptor) functionalized NPs. The nanoplatform was based on MnO_2 nanocrystals functionalized with was loaded with Protoporphyrin (ppIX), a sonosensitizer for sonodynamic therapy [66].

6. Cells as Trojan horses for drug delivery to GBM

The ability of different cell types to cross the BBB allow their use as Trojan horses carrying nanotherapeutic compounds to intracranial tumors. Several cell types have been used for this purpose (Table 4).

a. Mesenchymal stem cells (MSC) that, when transduced with viral vectors to express therapeutic proteins, have exhibited excellent tumor tropic nature and efficiently induced apoptosis in multiple GBM cell lines both in vitro and in vivo [67]. Thus, MSC were transfected to express human tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and were able to migrate into U87 brain xenografts in mice suppressing tumor progression [68]. Moreover, human placenta-derived mesenchymal stem cells

transduced with NK4 (an antagonist of hepatocyte growth factor) demonstrated that preferably migrate into GBM and inhibited growth of tumor cells inducing apoptosis [69].

b. Adipose tissue-derived stem cells that have innate tumor-homing ability[70].

c. Neural stem cells (NSCs). Loading of NSCs with mesoporous silica nanoparticles conjugated to ^{111}In allowed visualization of actively migrating NSCs toward GBM xenografts in real time [71]. NSCs derived from human pluripotent cells transfected with the HSV-TK/GCV (herpes simplex virus thymidine kinase/Ganciclovir) suicide gene system increased the survival time of mice bearing GBM xenografts [72].

d. Tumor-associated macrophages (TAMs). Tumor progression is accompanied by accumulation of TAMs which are usually located in the tumor stroma and have tropism towards the hypoxic areas of tumor. They can reach about 40% of tumor cell mass in GBM [73,74], so they can be used as carriers for chemotherapeutics for GBM. Thus, monocytes loaded with DOX-nanodiamonds were able to maintain viability after entrapment of nanoparticles, withhold their cargo until reaching tumor cells, target GBM cells and deliver DOX to induce tumor cell damage in mice-bearing orthotopic human GBM xenografts [75].

7. Conclusion

During the last two years marked developments have taken place in the application of nanomedicine to GBM therapy. The main area of research has been focused in implementing the use of different ligands to specifically targeting pathways that are actively involved in either BBB crossing to improve the pass of nanocarriers and their therapeutic payload to the brain and to deliver specifically the therapeutic cargo to either GBM tumoral cells or the tumor microenvironment. However, besides the progress, no clear information about the nanoparticle key critical design parameters that might facilitate BBB crossing is available yet. The dismal prognosis of GBM has also prompted the development of new therapeutic approaches, using methods based on the physical properties of nanoparticles as hyperthermia, photodynamic or photothermal therapies. The development of an efficient therapy against GBM based on nanoparticles require more knowledge at two levels: cellular and molecular biology

of the tumor to identify the more appropriate therapeutic targets and on the molecular determinants ruling both nanoparticle BBB crossing and selective targeting and cargo delivery to GBM.

8. Expert opinion

Glioblastoma multiforme is the most common and the most aggressive primary brain tumor, with a very poor prognosis, besides providing the best possible treatment, leading to a median survival of 14 months and minimal survival expectations at 5 years. This dismal prognostic has turned research towards nanomedicine as a new therapeutic approach that can deliver new therapeutic compounds or standard antitumoral drugs to GBM. This approach is based on the use of nanocarriers as versatile platforms that can incorporate ligands on its surface with different purposes such as increasing bioavailability of antitumoral small drugs by facilitating BBB crossing, selectively delivering therapeutic molecules to glioblastoma by targeted delivery through specific receptors which would deliver encapsulated cargoes (chemotherapeutic drugs, therapeutic biomolecules or molecules for diagnosis) directly to GBM cells which would decrease antitumoral-drugs-related side-effects, protect the cargo from degradation, and decrease immunogenicity.

To become efficient agents in GBM therapy, enhancing their chances to reach the clinical setting, nanocarriers have to increase the therapeutic cargo delivery to GBM tumor by fulfilling, at least, two sequential steps:

a. Overcoming the main biological barrier, BBB which stands between the blood stream and GBM tumor. So far, no clear knowledge of the main molecular factors involved in efficient nanocarriers crossing of BBB is available. This lack of information is further complicated by the limited knowledge about the preferential intracellular fate of a given nanoparticle, and its cargo, depending on the endocytosis pathway followed for cell entry. A better knowledge of these mechanisms would help to design a more efficient nanoparticles allowing them to efficiently cross the BBB while protecting its cargo from release and degradation. The general approach to achieve this objective has consisted in decorating the nanocarriers with targeting ligands to mainly endothelial cells as key components of BBB. Many different ligands have been used for this purpose, as it is indicated

above in this review, although no general rules have been issued on which targets provide most efficient BBB crossing for the nanoparticles and its cargoes. This lack of general rules allowing the design of highly efficient nanoparticles, has prompted the use of physical methods, like focused ultrasounds, to transiently disrupt BBB integrity. This can increase nanoparticle BBB crossing but represents a potential risk in the clinical setting since BBB opening achieved by this method is non-selective which might allow passing into the CNS of toxic and pathogens with an increased risk of toxicity and infections. Increasing nanocarrier BBB crossing is a key element in the development of a Nanomedicine-based therapy for GBM since it would increase BBB crossing for their cargo. This is especially relevant if we consider that temozolomide, a gold standard drug for GBM patients treatment, cerebrospinal fluid concentrations only reach 15 to 30 % of plasma concentrations and, for instance, increasing TMZ delivery to CNS could markedly improve its anti-GBM efficacy.

b. Selective delivery of the antitumoral cargo to GBM tumors. This is a critical step for achieving efficient nanoparticle-mediated GBM therapy. A great deal of work is being developed using ligands for different molecules that are overexpressed in GBM cell surface or on neurovascular endothelial cells as is the case for αvβ3 integrins. This approach is very promising and might lead to an accumulation of nanocarriers and its therapeutic cargo into GBM tumors increasing the efficacy of the therapy. A significant number of ligands have been used for this purpose, although it is difficult to define which ones of those ligands offer the highest accumulation of nanocarrier and cargo in GBM tumors since the nanocarriers bearing them are chemically very distinct entities which difficulties comparison. An alternative to selective delivery to GBM cells consists in targeting the tumoral microenvironment using specific ligands for either the extracellular matrix or the perivascular space. This approach can be used together with targeting specifically GBM cells. However, both approaches, targeting GBM cells and tumor microenvironment, approaches should be combined with the incorporation of a ligand to facilitate BBB crossing which significantly difficulties the chemical synthesis of the nanocarriers. This represents an additional problem since the optimal approach would require a sequential functionality of the nanoparticle: an initial one facilitating BB crossing and a second one allowing selective delivery of

the therapeutic molecules to GBM cells. This sequential approach might require a layer structure in the nanoparticle where, once the initial function (BBB crossing) has been achieved, that layer will be detached from the nanoparticle exposing the second layer designed to selectively target GBM cells. Synthesizing such particles is not easy to achieve, although different steps are being made in this direction.

The dismal prognosis of GBM patients, besides state-of-the-art therapy, has prompted the appearance of a significant number of additional therapeutic approaches beyond the antitumoral small drugs or nucleotide-based molecules such as miRNA or siRNA. These other therapeutic possibilities include hyperthermia using paramagnetic nanoparticles, light pulses-based (photodynamic, photothermal) or sound-based therapy as sonodynamics. However, the same general problems, BBB crossing and reaching selectively GBM cells, as indicated above still remain to be solved for these approaches.

During the coming years, a more successful design of nanocarriers for efficient BBB crossing and delivery of diagnostic and/or therapeutic molecules to CNS will require a significant effort improving chemical architecture of nanocarriers, likely through a more precise knowledge of the interactions among the different critical design parameters of nanocarriers including, among others, chemical composition, size, shape, and charge. However, to reach the point of finding a very efficient and reliable nanocarrier effective against GBM, more information about several aspects of GBM biology must be obtained including a precise knowledge of the role of key signaling molecules in GBM proliferation and survival. Other issues that should be approached in the following years include design of an effective combination in the same nanocarrier of BBB crossing abilities and selective targeting and delivery to GBM tumoral cells, and to establish the role of tumoral microenvironment on noncarriers functionality.

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Table 1. Summary of the ligands used, target, therapeutic load, and effect of the different nanoparticles used to target glioblastoma xenografts by parenteral administration.

Nanoparticle /strategy	Ligand	Target	Drug	Conclusion	Ref.
PEG-liposomes (phosphatidylcholine, cholesterol and DSPE)	Cell penetrating peptide CB5005	BBB penetration	Doxorubicin	Inhibition of Nuclear Factor kB. Prolongation of survival time if GBM-bearing nude mice.	6
LRP-1 targeted vesicles	Angiopep-2	LRP-1	Chaperones protein toxin	Higher brain penetration capability than other proteins	7
LRP-1 targeted vesicles	ANG4043 peptide	LRP-1	Paclitaxel	Clinical trials	8,9
Liposomes	Transferrin, specific peptides or monoclonal antibodies	TfR	Several drugs	In vivo anti-GBM activity	10
Phosphatidylcholine-cholesterol-PEG-DSPE liposomes	*p*-aminophenyl-α-D-mannopyranoside	GLU1	Curcumin and quinacrine	Promote penetration through the BBB into the brain. Target selected intracerebral regions.	11
Gold NPs	pH-responsive short tripeptide (Lys-Phe-Gly)	NGF	Doxorubicin	Tumor growth decreas in a BT-474 cell xenograft model	12

Liposomes, focused ultrasounds, circulating microbubbles	Ligand targeting MGMT	MGMT	MGMT inactivator (O6-(4-bromothenyl)guanine	Reduced tumor growth, prolonged survival of glioma-bearing mice	14

Table 2. Summary of the nanoparticles and targeting ligands described in section 3.2 used for actively targeting glioblastoma xenografts.

Nanoparticle /strategy	Ligand	Target	Drug	Conclusion	Ref.
PEGylated poly(L-γ-glutamylglutamine) liposomes	RGERPPR peptide	NRP-1	Paclitaxel	Higher cytotoxicity towards tumor cells in comparison with non-targeted liposomes	16
Niosomes	tLyp-1	NRP-1	Curcumin and doxorubicin	Higher toxicity levels than free drugs	17
PEG-PLGA NPs	DA7R (glioma-homing heptapeptide)	NRP-1	-	Also binds to the VEGFR-2 receptor	18
Liposomes	iNGR peptide	NRP-1	-	Deep penetration into GBM tumor and bind to its receptor NRP-1	19
PEG-PLA polymers	FHK peptide	NRP-1, Tenascin-C	-	Targeting intracranial U87 xenografts in mice	20
Iron oxide nanoworms and metallic silver NPs	octameric homing peptide PL3	NRP-1, Tenascin-C	Proapoptotic D(KLAKLAK)2 peptide	Accumulation in TNC-C and NRP-1-positive areas in clinical tumor samples	21
KB004, anti-EPHA3 monoclonal antibody	nonfucosylated IgG1j monoclonal antibody	EPHA3	-	Phase I clinical trial	22

PEG-PLGA NPs	Ki16425 is an inhibitor of LPA signaling	LPAR1	-	Inhibition of tumor progression in vivo	23
Immunoliposomes	anti CD133 antibodies	CD133	Temozolomide	Tumor size and an increased in median survival time	24
Cu and Se-loaded PEG-PEI copolymers	HER2 antibody	HER2	-	*In vivo* showed delivery of nanoparticles to the brain	25
Serum albumin and polycaprolactone NPs	anti-EGF antibodies	EGFR		Tumor growth inhibition in xenografts in nude mice	26

Nanoparticle /strategy	Ligand	Target	Drug	Conclusion	Ref.
2-methacryloyloxy-ethylphosphoryl-choline-based NPs	cMBP peptide, Inherbin3	MET, EGFR	Temozolomide	attenuate DNA damage repair and enhance TMZ sensitivity	27
Lipid NP	Anti PD-L1	PD-L1	Dinaciclib	Extended survival of mice in glioma models in combination with radiotherapy	28
PLGA-b-PEG copolymer	Anti-PDGFRβ aptamer	PDGFRβ	Dactolisib	BBB accumulation and mTOR inhibition	30
Tetrahedral framework nucleic acid NP	GS24 aptamer	Transferrin	Temozolomide	Overcame the resistance of TMZ, BBB crossing of mice	31
Gold NPs	U2 aptamer	EGFRvIII	-	Prolonged survival time of GBM-bearing mice	32
PEG nanomicelles	Borneol	P-glycoprotein	Doxorubicin	Tumor growth and metastasis inhibition of GBM models in mice	33
Hyaluronic acid-modified polymer	HRK-19 peptide	CD44	Docetaxel	Cross the BBB with negligible systemic toxicity and enhanced therapeutic efficacy	34
Iron oxide nanoworms, silver and albumin NPs	LinTT1 peptide	Cell surface p32	Paclitaxel	Doubling the lifespan of the mice in an aggressive orthotopic stem cell-like xenograft	35
Silver NPs/liposomes	Cholotoxin	MMP-2	-	Enhancement of radiptherapy effects	36

PEG-PCL micelles or PEG-DSPE liposomes	Cyclic RGD peptide	αvβ3 integrins	Doxorubicin	Survival time prolonged in GBM bearing mice	37, 38
PLGA NPs (intranasal)	Cyclic RGD peptide	αvβ3 integrins	Paclitaxel	Control the tumor burden (75 % reduction)	39
Gadolinium oxide NPs stabilized with PAC polymers and albumin	RGD dimer and lactoferrin	αvβ3 integrins	-	Enhancing radiation therapy effectivity in GBM xenografts	40

Table 3. Summary of different therapeutic approaches used in experimental models of glioblastoma xenografts.

Nanoparticle /strategy	Ligand	Target	Therapy	Conclusion	Ref.
Gold iron oxide NPs coated with β-cyclodextrin-chitosan	Adamantane-PEG-T7 peptide	GBM cells	antimiR-100 antimiR-21 Temozolomide	Presensitization of GBM cells to the systemically delivered temozolomide	45
Cationic polymeric NPs	Angiopep-2 peptide	Polo-like kinase 1, VEGFR-2	Anti polo-like kinase 1 and anti-EGFR-2 siRNA	Dual siRNA transfection	52
Solid lipid NPs	iRGD peptide	EGFR and PD-	Anti EGFR and PD-L1	Decrease in glioblastoma growth and	53

		L1	siRNA	prolonged mouse survival after radiation therapy	
PLA-PEG NPs	-	Histone deacetylases (HDCA)	Quisinostat (HDCA inhibitor)	Slow orthotopic GL261-induced GBM tumour growth and prolong survival of treated mice	54
Gold NP coated with polydopamine	-	Heat shock protein A5 (HSPA5)	Pifithrin-μ (HSPA5 inhibitor)	Improved radiotherapy and photothermal therapy efficiencies	55
Magnetosomes NPs covered with PLL	-	-	Hyperthermia	The tumor fully disappeared in all the mice bearing intracranial U87 GBM xenograft	57
SPIONs coated with silica mesolayers and carbon shells	-	-	Hyperthermia	Crossing the BBB and tumor death	58
DSPE-PEG$_{2000}$ - magnetic iron NP	RGDyK peptide and glucosamine	Neovascular endothelium and tumor cells	Hyperthermia	tumor-suppressive effect	59
Silica NPs, $ZnFe_2O_4$ core	iRGD peptide Sodium nitroprusside, DCC	-	Hyperthermia	Selectively ablation of tumor cells via magnetic hyperthermia	60
Casein	-	-	Photodynamic therapy:	Activation of the apoptosis cascade, cell cycle	63

nanoformulation			indocyanine green (photosensitizer) and genistein (PTK inhibitor)	arrest and damage the mitochondrial membrane potential of GBM cells.	
Hybrid metal-organic NPs	-	-	Photothermal therapy, GSH-responsive activation	Enhanced photodynamic therapy efficacy through intracellular MnO_2 reduction	64
Serum albumin NPs conjugated			Sonodynamic therapy, N-chlorin e6	Reduction in tumor size and increased animal survival	65
MnO_2 nanocrystals	Holo-transferrin		Sonodynamic therapy, Protoporphyrin	Suppression of tumor growth in a C6 tumor xenograft model	66

Table 4. Cells as Trojan horses for drug delivery to Glioblastoma xenografts.

Vector	Ligand	Target	Therapy	Conclusion	Ref.
Mesenchymal stem cells (MSC)	-	-	Cells transfected to express necrosis factor-related apoptosis inducing ligand	Tumor progression suppression in U87 xenografts in mice	68
Human placenta-derived MSC	-	-	Cells transfected with NK4	Tumor growth inhibition and apoptosis induction	69
Neural stem cells loaded with mesoporous silica NPs	-	-	Cells transfected with the HSV-TK/GCV suicide gene system	Survival increase time of mice bearing GBM xenografts	72
Monocytes-nanodiamonds	-	Tumor-associated macrophages	Doxorubicin	Doxorubicin delivery to induce tumor cell damage in mice-bearing orthotopic human GBM xenografts	75

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Figure legends

Figure 1. Scheme showing the structure of the BBB where all the components of BBB can be found.

Figure 2. Schematic representation of all the main pathways for a nanoparticle and its cargo to get into the cells.

Figure 3. Cartoon representing a nanoparticle with several possible ligands that can decorate the nanoparticle to direct it toward its target as well as the different loads it can take. On both sides of the general physicochemical properties as well as the different types of nanoparticles are represented.

Figure 4. Scheme representing the siRNA and miRNA endosomal escape. A model where siRNA is bound to a positively charges nanoparticle is depicted. Both receptor-mediated endocytic internalization mechanism and endosome escape are represented.

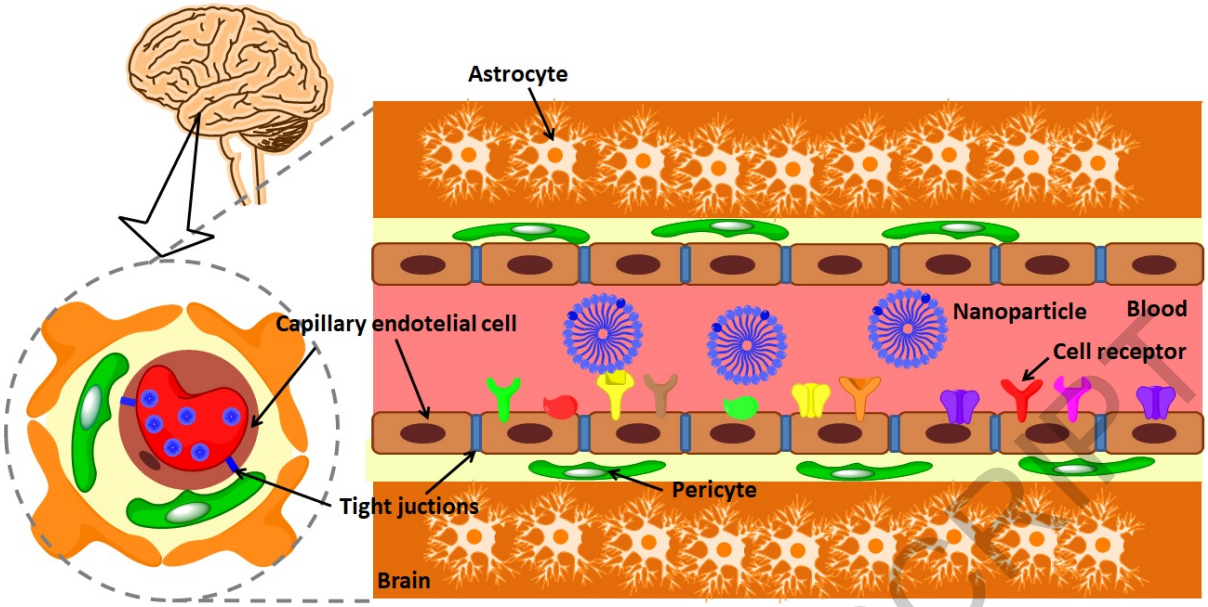

Fig 1

Fig 2

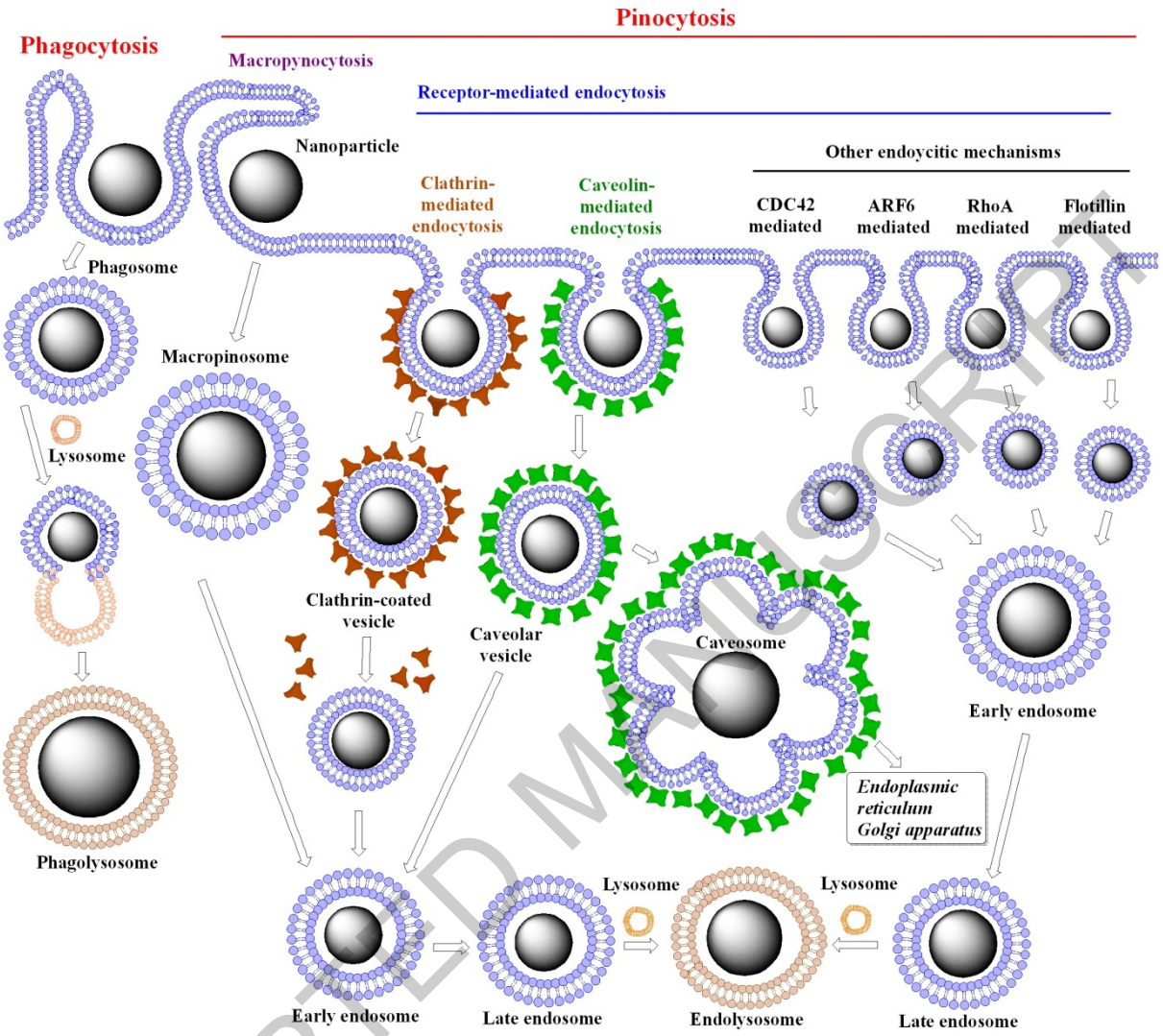

Fig 3

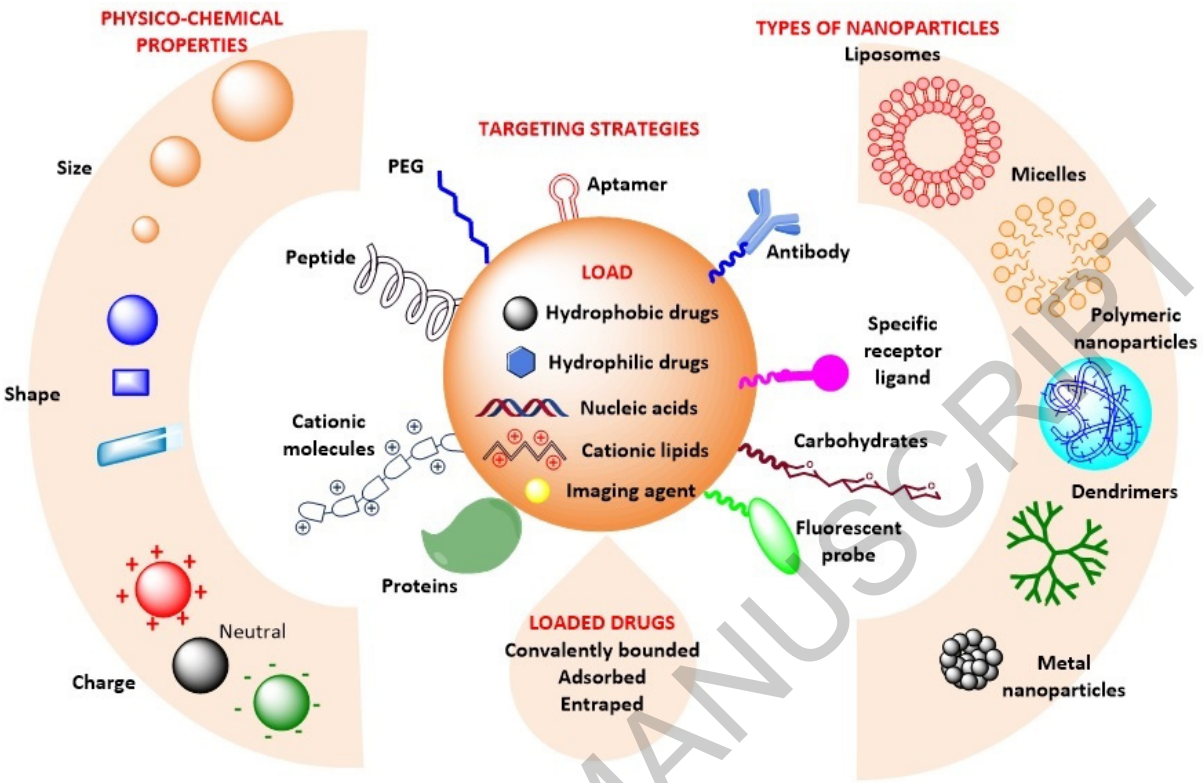

Fig 4