Chapter III.11

Nanotechnology-based approaches in glioblastoma treatment: how can the dual blood-brain/tumor barriers be overcome?

Matteo Moschetta[1§], Martina Trevisani[2§], Valentina Castagnola[2,3*] and Mattia Bramini[4*]

[1] *Center for Nano Science and Technology, Istituto Italiano di Tecnologia, Via Giovanni Pascoli 70/3, 20133 Milano, Italy*
[2] *Center for Synaptic Neuroscience and Technology, Istituto Italiano di Tecnologia, Largo Rosanna Benzi 10, 16132 Genova, Italy*
[3] *IRCCS Ospedale Policlinico San Martino, Largo Rosanna Benzi 10, 16132 Genova, Italy*
[4] *Department of Cell Biology, Faculty of Sciences, University of Granada, Fuente Nueva s/n, 18071 Granada, Spain*

§ These authors contribute equally

Abstract
Glioblastoma is one of the most common and aggressive tumors of the central nervous system, with an annual incidence of 3.2 every 100.000 persons. Successful glioblastoma treatment remains challenging despite a tremendous research effort, presenting a high recurrence ratio and a median survival rate of about 15 months after diagnosis. The present chapter provides an overall description of the current glioblastoma treatments, including a brief description of the classical radio-chemotherapy approaches. The issue of blood-brain barrier translocation, one of the most challenging limitations for pharmacological treatment of brain cancers, is also treated, exploring how nanotechnology can be a real game-changer in this area. The main body of the chapter focuses on describing a plethora of nanoformulations (synthetic, biological, and hybrid) as innovative and salient theranostic tools to actively cross the blood-brain and blood tumor barriers, target the glioblastoma, and deliver therapeutics. Finally, we draw some considerations on the future of these promising nanotechnology-based therapeutic approaches for the effective treatment of glioblastoma.

Keywords – glioblastoma, nanotechnology, biomaterials, drug delivery, blood-brain barrier, nanoparticles

1. INTRODUCTION

1.1 Glioblastoma multiforme occurence

Glioblastoma multiforme (GBM) is one the most common and lethal tumor of the central nervous system (CNS), part of a heterogeneous group of malignant neoplasms derived from glial cells. In western countries, it represents 16% of all brain neoplasms and 45.6% of the primary malignant ones [1,2]. The annual incidence of GBM is 3.2 per 100,000 with a minimum peak in children (0.15 per 100,000) and a maximum peak in 75-84 years old patients (15.03 per 100,000) [1–4]. Despite the progress obtained with the recent introduction of immunoglobulin therapy, the prognosis for patients with GBM remains poor, with a survival rate of 42.4%, 17.7%, and 5% after 6 months, 1 year and 5 years, respectively, and a median survival of 15 months after diagnosis [1,4,5]. GBMs are classified as primary (de novo) if the neoplasms arise without a known precursor; or secondary if GBM originates from another low-grade tumor. 61% of the total primary GBMs occur in the brain: 25%, 20%, 13%, and 3% at the level of frontal, temporal, parietal, and occipital lobes, respectively. Nevertheless, GBMs could also appear in the cerebellum and the spinal cord [6]. Besides age, other risk factors need to be taken into consideration in the development of GBMs. In particular, GBM incidence is higher in males than females (1.6:1) and in white people than blacks (2:1) [2,7,8]. Finally, less than 1% of patients affected by GBMs present comorbidity with hereditary syndromes, including types 1 and 2 neurofibromatosis, Turcot syndrome, and Li–Fraumeni syndrome [9]. Initially, only glial cells were considered the origin cells of GBMs; however, recent evidence demonstrated that diverse cell subtypes could be involved in the GBM occurrence. These cells originate during the differentiation process of neuronal stem cells into neurons and/or glia. They maintain similarities with neuronal stem cells with phenotypic variations, usually determined by genetic alterations in signalling pathways [10].

1.2 Pathogenesis of glioblastoma

The pathogenesis of GBMs has not yet been fully elucidated despite a large amount of research in the field. Nonetheless, its understanding remains a pivotal step in the development of effective therapies aimed at treating this disease. In particular, the identification of genetic markers involved in different steps of the GBMs occurrence is necessary for the development of targeted chemotherapeutic agents [11]. Several pathways have already been identified to be involved in the pathogenesis of glioblastomas. Mutations in genes related to metabolites transformation are frequently associated with GMBs occurrence. For instance, isocitrate dehydrogenase (IDH) gene mutations are reported in the majority of the cases of secondary glioblastomas (and rarely in primary ones) [12]. Typically, the identified mutations lead to a significant decrease of the binding affinity of IDH for isocitrate, preventing isocitrate conversion in α-ketoglutarate (α-KG). In addition, the reduction in binding affinity for isocitrate is accompanied by an enhancement in binding affinity for NADPH, with a consequent increase in 2-hydroglutarate (2-HG) formation. The accumulation of 2-HG is considered responsible for cancerogenesis [12,13]. Another metabolic protein involved in the oncogenesis of GBMs is the acid ceramidase (ASAH1). ASAH1 is an enzyme able to metabolize ceramides in sphingosine and free fatty acids. Under physiological conditions, ceramides promote senescence and apoptosis, while sphingosine boosts cell proliferation [14]. Modifications in ASAH1 level are found in GBM patients; here, the tumoral mass

benefits from ceramides/sphingosines imbalance, reducing apoptosis accuracy and enhancing tumoral cell spread in the tissue [14,15]. In recent years, ASAH1 inhibitors (such as carmofur, N-oleoylethanolamine, and ARN14988) have been tested in experimental studies because of their intrinsic properties in promoting cell death and consequently reducing tumor mass [16].

Intracellular pathways related to cell growth and differentiation are usually mutated in patients affected by GBM. It is reported that the Notch receptor family plays an important role in the development of primary GBMs. Notch signalling promotes cell proliferation during neurogenesis. Notch activity is regulated with a negative feedback process by Numb that promotes neural differentiation. Mutations at the level of the four Notch receptors (Notch-1,-2,-3, and -4) are associated with an increased glioma progression and aggressiveness [17]. In fact, inhibitors of Notch proteins, like α- and γ-secretase inhibitors, are successfully used in conventional drug therapy [18]. Also, sonic hedgehog gene (SHH) mutations are involved in glioblastoma occurrence. Under physiological conditions, SHH glycoprotein binds and inactivates the protein Patched1 (and other co-receptors), inducing the inactivation of the protein Smoothened (SMO). It is reported that abnormal activation of SHH signalling leads to the transformation of adult stem cells into glioblastoma stem cells [19]. Mutations are involved specifically either in the inactivation of Patched 1 and/or overactivation of SMO. In detail, when SMO is active, glioma-associated (GLI) transcription factors enter the nucleus, inducing the activation of GLI1 and GLI2 transcription factors. The transcription factor activation is responsible for cell proliferation, self-renewal, and epithelial-to-mesenchymal transition. Currently, SMO inhibitors, such as vismodegib, trametinib, and glasdegib, are under consideration for GBM pharmacological treatment [20,21]. Finally, mutations in growth factors genes might also be responsible for the occurrence and the progression of several types of neoplasms. Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and receptors of the epidermal growth factor (EGFRs) are frequently overactivated in GBM patients. VEGF favours GBM mass survival and progression by promoting a new and complex vascular net essential for tumor progression. PDGF and EGFR overexpression, mutations and/or rearrangements induce DNA synthesis, cell proliferation, migration, and adhesion. Their mutations are often associated with higher-grade GBMs. Anti-VEGF inhibitors (i.e., bevacizumab) are successfully used in combination with radioimmunotherapy in GBM treatment [22], while PDGF receptor inhibitors are able to increase tumor sensitivity to radio- and chemotherapy [23]. At last, OSI-774, an EGFR-TK inhibitor, is considered a promising drug for the GBM treatment by inducing apoptosis in both malignant primary and secondary GBMs [24].

1.3 Current treatments for glioblastoma

Despite the huge amount of research and efforts, GBM treatment remains one of the most challenging tasks in clinical oncology [25]. During the last decades, a large variety of possible innovative treatments were investigated with limited success. The current standard therapy includes surgical resection followed by radiation and pharmacological treatment with temozolomide (TMZ) (Temodar) and an oral alkylating agent [6,25,26]. However, surgical resection is a demanding and invasive procedure. Frequently, GBMs are present in a delicate area of the brain difficult to reach, including somatosensory and motor cortex and, for this reason, clinical surgery is not completely curative, needing a complementary pharmacological treatment [27]. Despite the progress obtained with the recent introduction of immunoglobulin therapy, the prognosis for patients with GBM remains poor, with a median survival of 15 months after the first diagnosis [1]. In the last 30 years,

liposomes have been investigated as a vehicle for anticancer therapy due to their intrinsic properties, such as their small size, low toxicity, and high biodegradability [28]. Liposomes are a promising tool for drug delivery due to their ability to encapsulate a large variety of biomolecules, from low molecular weight compounds to peptides and proteins, protecting them from degradation. As a matter of fact, liposome-based antifungal and anticancer drugs such as AmBisome and Doxil have been approved for clinical treatments [29]. Furthermore, standard approaches include not only anti-tumor therapies but also strategies aimed at providing supportive care to patients. We should not forget that GBMs are aggressive tumors, associated with a large variety of complications disorders. Typically, patients present secondary pathologies induced both by the presence of the tumor itself and/or by the side effects of pharmacological therapy. Myelosuppression, nausea, fatigue, alopecia, anorexia, cerebral edema, seizures, cognitive impairment, and mood disorders are some of the most common comorbidities that GBM patients might face [30–34]. Secondary neoplasm-related symptoms and therapeutical side effects are usually treated pharmacologically. For instance, Levetiracetam, an anti-epileptic drug, is successfully prescribed for patients with seizures because of its high tolerability and the absence of interaction with chemotherapeutic agents [35].

1.3.1 Surgery

Surgical resection is the standard procedure for GBM treatment. However, complete resection of the tumoral mass is often difficult because of its invasiveness rate and localization in sensitive areas of the brain. For example, tumors localized into the eloquent cortex or basal ganglia are not accessible to surgical resection, with a consequent worse prognosis for patients [25]. In addition, associative areas that control speech, motor function, and senses are usually niches of GBM cells, impossible to be removed. In this scenario, radical resection of the primary tumor mass is not curative and infiltration of tumoral cells frequently leads to later disease progression or relapse associated with a significant reduction of patient survival rate [3,27]. Furthermore, tumor recurrence depends on the margin of the original lesion. Tumors with a diameter greater than 5-6 cm are associated with a worst prognosis [7]. In addition, 80% of the relapse cases occur within 2-3 cm of the margin of the original lesion, and a greater extent of resection improves treatment outcomes, preserving brain function and quality of life [36]. During the last decade, functional magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) in preoperative planning have been introduced to improve surgery efficacy despite their evident limitations (i.e., cost, need of specialized equipment, and operators). The use of 5-aminolevulinic acid (5-ALA) dye for fluorescence guidance was shown to be more effective compared to the conventional neuro-navigation-guided surgery alone [37]. However, independent reviews displayed only low-quality evidence of 5-ALA or DTI neuro-navigation efficacy in the complete tumor resection in patients with high-grade glioma [38].

1.3.2 Radiation Therapy

Traditionally GBM surgical resection can be coupled with radiotherapy (RT) in an attempt to completely withdraw the tumor mass. Stereotactic radiosurgery demonstrates to be effective in improving life expectancy and in reducing the probability of relapse in patients with high grade and early diagnosed GBM [39]. However, RT includes several risks and limitations, i.e., transient and permanent neuronal damage, necrosis, endocrinopathy, and leukoencephalopathy [40]. Until 2005, the standard treatment required RT alone, when Stupp *et al.,* published the results of phase III clinical

trial in which they demonstrated that RT in concomitance with TMZ chemotherapy was more effective than RT alone [26]. In this study, the survival rate of patients that received RT associated with TMZ was of 14.6 months compared to 12.1 months of patients who did not receive TMZ. The positive outcome was confirmed in the long-term survival rate: 27% of patients that receive TMZ were alive after 2 years and 10% of them after 5 years (compared to 11% and 2% of whom receive RT alone) [41]. TMZ was administered concurrently with RT at a dose of 75mg/m^2 daily for 6 weeks. After 1 month of resting period, TMZ administration was increased (150 mg/m^2) for 5 days for the first month. If tolerated, the dose was then increased at 200 mg/m^2 for 5 consecutive days per month [42]. Nonetheless, the effectiveness of Stupp therapy is dependent on the genetic background of the patients. The methylation of methyl guanine methyl transferase (MGMT) gene is a predictor of a positive outcome. MGMT is a critical DNA repair protein that protects tumor cells against alkylating chemotherapeutic agents [43]. TMZ is able to methylate DNA at the N7 and O6 position on guanine, which leads to the failure of DNA miss-match repair system and a consequent blockage of the cell cycle [39]. However, it has been reported that high levels of MGMT activity in tumor cells are associated with poor TMZ response. Since MGMT gene encodes for a DNA repair enzyme, patients with methylation (inactivation) of the gene are more subject to DNA damage. TMZ is a genotoxic alkylating agent that damages the DNA inducing cell apoptosis; indeed, patients with an overactivation of MGMT are refractory to TMZ therapy [41].

1.3.3 Tumor-treating fields therapy

Tumor-treating fields (TTFields) are an innovative non-invasive therapeutic approach based on the transcutaneous delivery of low-intensity electrical fields to tumor cells, with the purpose of disrupting neoplastic mass, interrupting cell division, and causing apoptosis **(Figure 1)** [44,45]. In general, TTFields modality consists of the transcutaneous delivery of an alternating electric field characterized by low-intensity (1–3 V/cm) and intermediate-frequency (100–300 kHz). TTFields are delivered by transducer arrays placed on the skin close to the tumor and act regionally and noninvasively. The electric field induces the polarization and the alignment of different proteins with a large dipole moment, inhibiting the proper protein polymerization and organization and consequently compromising cellular activity [44]. In addition, it has been proposed that the electric field can affect a larger variety of cellular processes, including DNA repair, autophagy, cell membrane organization, and blood-brain barrier (BBB) permeability, increasing the efficacy of chemotherapeutic treatments. The efficacy of TTFields treatment is dependent on the frequency, power intensity, and duration of the stimuli. Low-frequency alternating fields (lower than 1kHz) are known to lead to the disruption of cell membrane polarization and alterations in neuronal cell excitability interfering with the action potential occurrence [46]. Initially, intermediate frequencies alternating fields (between 100 to 500 kHz) were not considered for GBM treatment due to the incapability of generatin enough thermal energy to significantly damage the tumor mass [47]. Nonetheless, recently, intermediate frequency fields revealed to inhibit cancer cell growth and proliferation both *in vitro* and *in vivo* by altering microtubules organization during mitosis [48]. High-frequency electric fields (more than 500 kHz) are effective in damaging tissue by locally increasing the temperature [49]. In 2015 a new device called Optune, that delivers TTFields, was clinically approved in association with TMZ treatment, demonstrating efficacy in increasing the survival rate for patients (specifically 20.5 months versus 15.6 months with TMZ alone) [50]. In 2019, a phase III (EF-14) clinical trial demonstrated that TTFields, in association with TMZ, significantly improved

the duration of progression-free and overall survival of patients affected by GBMs independently of other prognostic factors. Patients treated with TTFields (plus TMZ) showed a median survival rate of 24.9 months and 5-year survival of 29.3% [51].

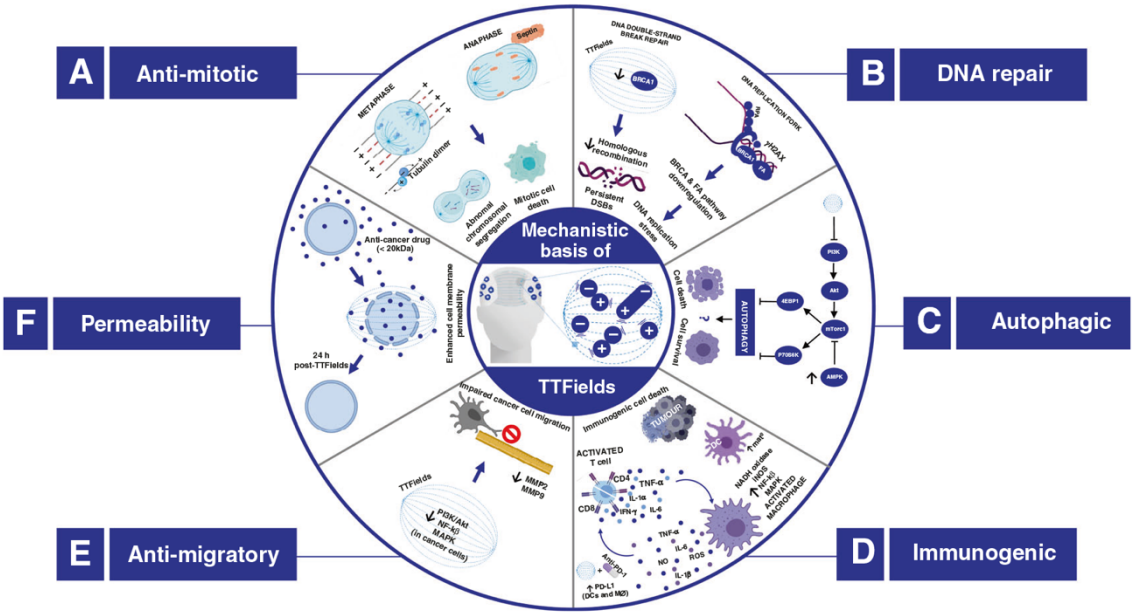


Figure 1. *Low-intensity, intermediate-frequency, alternating electrical fields exert biophysical forces on a variety of charged and polarisable molecules to elicit a spectrum of biological effects.* ***A Antimitotic effects****: during metaphase, the electric fields are uniform, causing dipolar molecules, such as tubulin, to align with the field. TTFields therefore interfere with tubulin polymerisation and depolymerisation during metaphase. At anaphase, TTFields prevent localisation of septin proteins to the mitotic spindle and inhibit assembly of the septin complex into a ring structure at the cleavage furrow. During cytokinesis, the electric fields are non-uniform, with the fields converging on the cleavage furrow, where the field intensity is the highest. As a result, strong dielectric force is applied on polarisable objects, pushing them towards the high-intensity region. Together, these effects result in abnormal chromosome segregation and/or cell death.* ***B DNA repair.*** *TTFields have been shown to downregulate BRCA and Fanconi anaemia (FA) pathway genes, which have been associated with increased replication stress and increased double-strand break (DSB) formation. Additionally, homologous recombination repair (HRR) is impaired by TTFields, resulting in reduced efficiency of DSB repair.* ***C Autophagy.*** *TTFields have been suggested to prevent the inhibitory effects of the PI3K/Akt/mTORC1 signalling pathway on autophagy, resulting in increased activation of autophagy with TTFields therapy. Further studies are needed to ascertain whether autophagy is activated as a cell survival or cell death signal in response to TTFields.* ***D Antitumour immunity.*** *TTFields stimulates macrophages (Mø) to secrete reactive oxygen species (ROS), nitric oxide (NO) and proinflammatory cytokines such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-6. Additionally, TTFields promote immunogenic cell death via dendritic cell (DC) recruitment and maturation (mat), ultimately leading to an increase in the accumulation of CD4 + and CD8 + T cells at the tumour site. The combination of TTFields with anti-PD-1 therapy might enhance PD-L1 expression in infiltrating DCs and macrophages to further enhance antitumour immunity.* ***E Anti-migratory.*** *TTFields reduce the capacity of cancer cells for migration and invasion through nuclear factor (NF)-κB-, mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K)/Akt-dependent mechanisms.* ***F Cell membrane permeability.*** *TTFields increase cell membrane permeability by increasing the number and size of holes in the cell membrane, thereby potentially enhancing sensitivity to chemotherapeutic drugs. Reprinted from (Rominiyi et al., 2021) under the terms of the Creative Commons CC BY license; Springer Nature 45.*

1.3.4 Chemotherapy

Chemotherapeutic treatment is commonly not administered alone because of its limited effectiveness, in particular in patients with a higher grade of GBM. To improve the efficacy of the chemotherapeutic agents, their administration is traditionally followed by radiotherapy [40,52]. Alkylating agents like TMZ, carmustine, or BCNU (bis-chloroethylnitrosourea), and lomustine (CCNU) are used to treat the majority of primary tumors after partial or total resection of the cancer mass [40,52]. BCNU and CCNU are administered rarely because of their remarkable side effects and their tendency to induce drug resistance when used to treat cancers at early stages [53]. Since 2005, TMZ is used as an adjuvant in concomitance with radiotherapy as standard treatment for almost all patients with GBM, although, because of its high price, its use is restricted to richer countries [52]. In addition, TMZ is also responsible for inducing many side effects [54,55]. Other chemotherapeutic agents like carboplatin, oxaliplatin, etoposide, irinotecan, procarbazine, and vincristine are alternatively used for patients who do not respond to the drugs mentioned above. Anti-angiogenic agents like anti-VEGF monoclonal antibodies (bevacizumab), anti-FGF antibodies, monoclonal antibodies targeting EGFR (erlotinib and gefitinib) and tyrosine kinase inhibitors have been proven to be partially effective against GBM [40]. In particular, bevacizumab is efficient in improving the progression-free survival of patients with recurrent GBM; however, no significant effect on the overall survival has been observed [56]. The use of CCNU is commonly avoided because of its relevant systemic toxicity [57]. However, CCNU can be used in the form of an implant in the resection cavity in order to overcome systemic toxicity and improve patients' survival rates [58].

Most of the drugs previously described for the treatment of GBMs are administrated orally or systemically and, despite their *in vitro* efficacy, do not manage to specifically reach the tumor site [59]. The difficulty in targeting the brain is principally due to the role of the BBB, which strongly limits the access of therapeutic and diagnostic agents into the brain. The BBB is composed of microvascular endothelium and neuroglial cells such as astrocytes, pericytes and microglia, which regulate the transport of substances from one side of the barrier to the other [60]. Several approaches have been designed to bypass the selectivity of the BBB, for example the controlled disruption of the barrier to increase permeability [61]. However, altering the integrity of the barrier, even temporarily, is a risky approach given that the probability that pathogens present in the bloodstream cross the barrier at the same time of the drug is high [62]. Functionalized liposomes, nanoparticles (NPs) and biomaterials have been designed to overcome these limitations. Their small size and their possibility to be functionalized to penetrate the BBB, without disrupting and compromising its function, is the reason for the growing scientific interest of recent years in nanomedicine.

2. NANOTECHNOLOGY FOR DRUG DELIVERY TO THE BRAIN

2.1 The blood-brain barrier

The CNS of developed organisms possesses specific biological barriers performing the fundamental task of regulating the brain microenvironment, allowing the entry of essential nutrients while stopping harmful substances, which is vital to maintain a healthy state. In particular, the main CNS interface barriers involve the endothelium of brain microvessels forming the BBB, the choroid plexus epithelium secreting cerebrospinal fluid (blood-CSF barrier), and the epithelium of the arachnoid mater (arachnoid barrier) that covers the outer brain surface [63]. Due to its large surface area (around 12-18 m^2 in a human adult), the BBB represents the most important biological surface of exchange and, as a consequence, it plays a pivotal role in the regulation of molecular trafficking across the CNS, and its activities are crucial to brain homeostasis [64].

2.1.1 Physiology of the BBB

The BBB is primarily constituted of specialized brain microvascular endothelial cells (BMECs) that create the walls of the non-fenestrated brain capillaries. BMECs display unique properties compared with those supplying blood to the rest of the body, allowing a tight regulation of ions, molecules, and cells movement between the blood plasma and the brain extracellular fluid. Indeed, due to the complex structure and low lipid permeability of this barrier, only gaseous molecules, such as O_2 and CO_2, and a few small polar molecules can freely and passively cross it, depending on their lipid solubility, molecular weight, and hydrogen bonding capacity [65]. Instead, larger molecules are transported across BBB through two possible pathways: transcellular and paracellular routes. The transcellular pathway is mediated by electrochemical and ATP-based active transporters present on the cell membrane. This route is mainly energy-dependent substance-specific and includes transcellular diffusion, receptor-mediated endocytosis, efflux, and carrier-mediated transport system [66,67]. On the other end, paracellular transport is passive and depends on the concentration gradient. It is regulated by specific transmembrane and membrane-associated proteins called tight junctions (TJs), which assemble into stable complexes increasing BBB tightness and modulating the transport between adjacent cells [68,69]. TJs involve at least three different families of proteins called claudins, occludins, and junctional adhesion molecules (JAMs). However, the effectiveness of the TJs seems to be related to the intracellular scaffolding proteins zonula occludens (ZO-1, ZO-2, and ZO-3) that bind claudins and occludins via cingulin to the cytoskeleton [70,71]. This tight paracellular barrier creates a polarized cell with two distinct membrane compartments suggesting that blood-to-brain movement can be regulated by controlling cellular transport properties [64].

Although endothelial cells constitute the barrier, the close association to astrocytes is also known to contribute to the induction of BBB properties [72–74]. Astrocytes are glial cells, which possess polarized cellular processes surrounding the vascular tube and secrete chemical agents acting on endothelial cells. In particular, they are able to enhance TJ levels by the secretion of several effector molecules, such as transforming growth factor-β (TGFβ), glial-derived neurotrophic factor (p), and basic fibroblast growth factor (bFGF). Together with pericytes, they also express angiotensin I, resulting in the strengthening of tight junctions and inhibition of transcytosis [74]. *In*

vitro co-cultures of endothelial cells and astrocytes or endothelial cells in astrocyte-conditioned media have been shown to lead to the formation of more complex TJs, elevated expression of transporters, and increased transendothelial electrical resistance [75]. Moreover, deficiency in BBB function in some neuropathologies that involve glia suggests that the presence of astrocytes is fundamental for the maintenance of barrier properties.

More recently, pericytes, neurons, and monocytes have demonstrated the possibility of inducing some barrier functions, clarifying the evidence of multiple cells and agents' involvement [74,76]. In particular, pericyte recruitment seems crucial to establishing BBB characteristics [77,78]. Indeed, it was shown that a complete loss of pericytes in platelet-derived growth factor (PDGFb) results in dysfunctional TJs and increased vascular permeability [75].

2.1.2 Strategies to overcome the BBB

Even though the BBB is essential for normal CNS function, at the same time, it is a daunting hurdle during therapeutic intervention. For that matter, emerging knowledge regarding BBB structure and function has paved the way to novel strategies to overcome this barrier, thus enabling brain drug delivery.

According to some estimates, BBB precludes from entering in the CNS almost all large molecule drugs and around 98% of small molecule therapeutics. In particular, it has been estimated that only 0.1% of intravenously administered therapeutics are able to reach the brain [79]. As a drawback, increasing concentrations of drugs are required to reach the target, meaning a higher potential systemic toxicity. Therefore, treating neuronal diseases remains pretty challenging, and several strategies have been explored to overcome the BBB and deliver the drug to the target site in the brain. These approaches could be divided into invasive and non-invasive approaches. Invasive approaches consist in using a direct administration route through intracerebroventricular (ICV) devices implanted under the patient scalp [80]. Although less invasive devices, such as polymeric implants, have been developed, they all carry problems related to poor drug penetration and unpredictable drug distribution [81]. Moreover, these approaches are associated with high intracranial pressures and local toxicity, leading to infections and brain trauma. An interesting recently developed brain cancer treatment makes use of so-called microMESH, meaning implantable degradable materials with the shape of a micrometric polymeric net able to wrap around the brain tumour mass [82]. The unique features allow microMESH to achieve a closer interaction with the tumour mass, increasing therapeutic efficacy. Its structure consists of two separate compartments in which different drugs can be loaded and released towards the tumour mass in an independent, precise, and prolonged fashion. Although the therapeutic results seem encouraging, this approach is rather invasive since it requires the placement of the microMESH through surgery.

Alternatively, non-invasive procedures currently in studies include chemical modification of drugs, which gives them a more lipophilic nature or conjugates them to specific receptor ligands [83,84]. Recently, virus-mediated delivery is gaining increasing interest due to its wide application and scalability [85]. Other approaches are (*i*) the exploitation of active BBB transporters by drug conjugation with a substance that enters via active transport mechanisms [86]; (*ii*) the transient and partial opening of the paracellular route by the modulation of TJs' efficacy, through chemicals [87], protein binding peptides [87,88] or light-sensitive NPs [89]; (*iii*) the administration through the intranasal route, using the connection between the trigeminal and olfactory nerves in the nasal mucosa to reach the brain [90]. This last possibility allows an easier administration, rapid action, and reduced

systemic side effects, even if it has some drawbacks such as short retention time and restrictions related to the nasal anatomy that ends in low therapeutic concentrations of the drugs reaching the brain. Finally, drug delivery could be performed through nanocarriers, such as polymeric or inorganic nanoparticles, liposomes, nanofibers, and micelles, which can be engineered to enhance their uptake in the brain [65,87].

Among these approaches, nanoparticles and nanomaterials (NMs) present several advantages such as low toxicity, high drug-loading capacity, good stability, and biodegradability. In addition, functional NMs can be engineered to react to external and internal stimuli, improving the control over the delivery process. For all these reasons, NMs are considered the most enticing and versatile drug delivery systems to the brain [91].

2.2 Nanomaterials for targeting and drug delivery

The term "nanomaterial" (NM) generally defines any particulate material whose internal or external dimensions lie in the size range of nanometres, frequently considering 100 nm as a delimiting size between the nanoscale and the microscopic levels. Thanks to their unique characteristics not found in their bulk counterparts, which include high surface-to-volume ratio, high surface energy, and excellent mechanical, thermal, electrical, optical, and magnetic properties, nanomaterials have been proposed as a way to increase delivery efficiency, decrease off-target effects and improve drug kinetics [92–94]. NPs have proven to be excellent carriers for therapeutic molecules that can be incorporated either within the bulk or anchored onto the surface [95]. NPs can be classified, according to their bulk material, into inorganic or organic (polymeric, biomimetic-based, carbon-based). Inorganic NPs, thanks to their broad range of physicochemical properties, high stability, and resistance to enzymatic degradation, represent a widely explored nanocarrier. Their intrinsic characteristics (electronic, optical, and magnetic properties) can be tailored by controlling their chemical composition, crystal phase, size, shape, and surface characteristics to make them truly beneficial for imaging and therapeutic purposes. However, toxicity might be an issue in some cases since they contain heavy metal atoms and often need a biocompatible coating on their surface [95]. Among inorganic NPs, silica nanoparticles (Si-NPs) are promising candidates for brain drug delivery thanks to their manufacturing controllability and relatively low cost. They can be easily modified by conjugating specific ligands on the surface in order to increase transport efficiency [96]. Moreover, mesoporous silica nanoparticles (MSNs) are also popularly used due to substantial specific surface area for loading drugs or ligands.

Gold NPs, especially in their ultrasmall size regime, have been widely employed as nanocarriers, anticancer, and imaging agents, thanks to their intrinsic biocompatibility and the almost endless possibilities of modulating their physical-chemical properties and decorating their surface [97–100]. Finally, silver, titanium dioxide, and iron oxide NPs have also been investigated to cross the BBB [95,101,102].

Polymeric nanoparticles present high flexibility in terms of possible composition, dimension, morphology, surface functionalization, and biodegradability. Polyethylene glycol (PEG), poly(lactic acid) (PLA), and especially poly(lactide-co-glycolic-acid) (PLGA) are three widely employed polymer-based carriers [103]. However, some issues encountered for traditional linear polymers, such as the low number of interaction sites and the relative drug-loading capacity, still restrict their applicability. In this optics, some purposely designed polymeric NPs with large specific surface areas have been introduced for drug delivery purposes. For instance, dendrimers are a type of special stretched polymers with much more accurate controlled structures to which a

large number of peripheral functional groups can be attached in order to improve the amount of drug loading areas [104].

Carbon-based NMs are constituted of carbon atoms, which can exist in different molecular forms entailing different properties. Carbon nanotubes, graphene, and fullerene are the most studied among carbon-based NMs. Brain drug delivery using carbon-based nanocarriers has been attracting increasing attention in recent years due to their high biocompatibility, high surface area (allowing an easy surface functionalization and high cargo capability), and unique physicochemical properties [95,105].

A last class of NMs is biomimetic-based nanocarriers consisting of either biologically endogenous particles, such as natural vesicles like liposomes and exosomes, or synthetic NPs covered by natural cell membranes (nanoghosts – NGs). These systems are, by definition, highly biocompatible, but the more substantial advantage of their use is that they retain their targeting ability in the biological environment and remain in the blood circulation for a longer time by escaping the immune system sequestration. This characteristic will be clarified in the next section in relation to the formation of the so-called "biomolecular corona". The biomimetic approach is cutting-edge and extremely promising, and a whole section will be devoted to it in this Chapter.

As acknowledged, morphology and surface chemistry greatly influence NMs properties. In particular, tuning properties such as shape, size, surface ligands, and charge might improve transport efficiency. The dimension of nanocarriers is one of the most important features for their transport across BBB, as it was shown that diameters around 30-50 nm could facilitate endocytosis within endothelial cells [106,107]. The shape of nanomaterials could influence the cellular uptake of drugs too. Although spherical nanoparticles are the most used in nanomedicine, non-spherical NPs (like discoidal particles, nanorods, and filamentous particles) seem to have better performances in tumor treatment in terms of their ability to avoid the uptake by macrophages in organs and vessels, cellular uptake, and biodistribution and their ability to cross biological barriers [108,109]. Moreover, due to the negatively charged cell membranes, the surface charge of nanocarriers represents another crucial factor for regulating brain drug delivery. Consequently, the internalization of neutral and negatively charged particles is much more difficult with respect to positively charged ones [110].

2.2.1 Biological interactions at the nanoscale: strategies to overcome targeting limitations

In virtue of their specific size, the living machinery processes all the nanocarriers through sophisticated endogenous cellular active pathways. These processes are mediated by the presence of the so-called "biomolecular corona", a layer of proteins, lipids, and other biomolecules that spontaneously adsorb onto the NMs surface when they enter in contact with biological fluids [111–113]. This layer, and not the engineered surface chemistry, defines the biological identity of NMs and drives the recognition processes at the nanoscale, hence their interactions with cells and the immune system and their final biological fate [114,115].

Therefore, nanostructures have unique access to key biological processes, but at the same time, their targeting capability can often be masked and diverted, making them susceptible to falling in the body's protective mechanisms and incapable of effectively reaching their target [116,117]. This frame strongly limits the translation of nanoformulations into clinical applications [118].

There are only two ways out of this paradigm. The first one, extensively explored, is trying to elude the biomolecular corona formation by shielding the NPs with appropriate coatings or using the ultrasmall size regime [119–121].

A second most recent appealing strategy aims at exploiting a corona of endogenous moieties (including but not limited to proteins, antibodies, cell membranes, and exosomes) to guide the biological crosstalk without further endogenous interference [122–125]. This approach is called biomimetic and will be treated later in more detail, specifically for GBM targeting and therapy.

2.3 Nanomaterials for glioblastoma treatment

2.3.1 BBB in pathological conditions

Thanks to its morphological and functional characteristics, the BBB represents an obstacle for brain drug delivery. However, BBB properties, such as its permeability, are often subjected to several changes in concurrence with pathological conditions of diseases such as stroke, seizures, hypertensive encephalopathy, traumatic brain injuries, multiple sclerosis, Parkinson's disease (PD), and Alzheimer disease (AD). For example, during an ischaemic stroke, BBB becomes hyperpermeable to macromolecules [126]. Similarly, in multiple sclerosis, loss of organization of junctional molecules in cholesterol-rich cell membrane regions leads to increase BBB permeability. Moreover, BBB permeability can be significantly changed by the disruption of adherent junctions [127]. In the presence of brain tumors, such as glioblastoma instead, a new type of barrier, called brain tumor barrier (BTB), is formed. It possesses heterogeneous properties, with high permeability in bulk tumour areas, while almost null permeability in peripheral regions and, in combination with BBB, it contributes to hindering drug delivery to the brain [128].

Patients affected by glioblastoma possess brain regions with intact BBB and others covered by the aforementioned BTB, with substantially different structural characteristics, however limiting the penetration of the drug delivery system. Moreover, glioblastoma induces the neovascularization of the brain since high volumes of nutrients and oxygen are necessary for tumour expansion. BTB abnormalities summed to the specificity of glioma result in an altered brain permeability [129]. In this scenario, it is possible to distinguish three different types of vessels: continuous non-fenestrated vasculature with a permeability similar to the normal one, continuous, fenestrated endothelial vasculature, and discontinuous endothelial vasculature whose drug permeability is dependent on the molecular weight of the diffusing therapeutics. Moreover, both BBB and BTB vasculature may express P-glycoprotein (P-gp) that is able to promote drugs efflux from the cells, thus preventing the delivery of most commercial therapeutics [130].

2.3.2 NPs for targeting of glioblastoma

Nanocarriers offer several advantages in terms of drug delivery for the treatment of glioblastoma. As described before, given the nanometric size, they have higher chances to penetrate the BBB thank to active recognition and trafficking. Moreover, they can reduce the systemic toxicity of anticancer therapeutics.

Among other parameters, size is a crucial factor to consider when designing NPs for glioblastoma targeting. It was shown that dimensions should be optimized according to the tumour

stage. During the early stage of progression, smaller NPs (less than 20 nm) are more likely to accumulate at the tumour site, while as the disease progresses, also larger NPs can penetrate the BTB [130].

Additionally, they can passively be accumulated into the tumour due to the newly generated defective vasculature and the restriction of lymphatic drainage, an effect known as the Enhanced Permeability and Retention (EPR) effect [130].

Even if several strategies to treat glioblastoma rely on the EPR effect, this is often not sufficient to ensure a high accumulation of nanocarriers in the tumour site since the permeability of tumour vessels may not be homogeneous. Moreover, the presence of a dense brain matrix that limits diffusion and the elevated interstitial fluid pressure contribute to reducing the efficiency of drug delivery based on the EPR effect [131]. A possible strategy to overcome these limitations consists in the active targeting of glioblastoma with the introduction of affinity molecules onto nanocarriers' surfaces, such as antibodies or peptides, that are able to bind to antigens of targeted tumour cells, thus increasing cellular uptake and accumulations [132]. In order to overcome both BBB and BTB, dual-targeting drug delivery systems based on receptor-mediated endocytosis were developed. One possible dual-targeting strategy consists of surface modification of nanocarriers through two different ligands, being one of which able to target the BBB, while the other targets the tumour cells [133,134]. Another possibility is to bind one single ligand that is overexpressed both on BBB and tumour cells, such as the low-density lipoprotein receptor-related protein (LRP). It was demonstrated that nanocarriers targeting LRP are able to cross BBB via LRP mediated transcytosis and are uptaken by glioma cells through LRP mediated endocytosis [135]. Xin *et al.* conjugated angiopep-2, a ligand for LRP, to PEG-PCL nanoparticles for the *in vitro* and *in vivo* targeting of tumour and BBB using U87 MG cells, 3D glioma tumour spheroids, and intracranial glioma mice model. They demonstrated enhanced antitumour efficacy with respect to free drugs [135,136].

Mu *et al.* proposed the conjugation of C-terminal telopeptide of type I collagen (CTX) to iron oxide NPs to achieve specific targeting. Indeed, CTX is able to bind matrix metalloproteinase-2 (MMP-2), which is overexpressed on many brain tumours' surfaces. Flow cytometry analysis of SF-763 and U-118 MG cells incubated with NPs showed that even if non-specific uptake was observed, CTX-conjugation enhanced the uptake of NPs by target cells by more or less 30% with respect to non-conjugated NPs [137].

Ramalho *et al.* demonstrated *in vitro* enhanced uptake by glioblastoma cell lines using PLGA nanoparticles conjugated with a transferrin receptor-specific monoclonal antibody (OX26), which is highly overexpressed on glioblastoma with respect to normal tissues. Cellular uptake of conjugated NPs by U251 cells after 0.5 h and 2 h of incubation was found to be 1.89 and 1.37-fold higher than unmodified NPs, respectively, while regarding U87 cells, the cellular uptake was 1.70 and 1.41-fold higher in respectively 0.5 and 2 h incubation. Moreover, in NHA cells, the cellular internalization of OX26-NPs increased with conjugation, exhibiting uptake rates 1.67 and 1.21-fold higher in 0.5 h and 2 h incubation, respectively [138].

Interleukin-13 receptor alpha 2 (IL-13Rα2) represents another possible target that can be exploited in this application as it is overexpressed in glioma cells compared to healthy ones. Gao *et al.* proposed the employment of PEGylated-PCL nanocarriers decorated with an IL-13 peptide ligand. *Ex vivo* and *in vivo* imaging demonstrated that the presence of IL-13 on nanoparticles' surface resulted in an increased tumour localization [139].

MiR-21 is an oncogenic microRNA and an apoptosis suppressor (miRNA) overexpressed in glioma cells compared to normal brain tissue. Seo *et al.* tested anti-miR-21-loaded PLA-

hyperbranched polyglycerol (HPG) nanoparticles, showing promising results in terms of targeting and antitumour effect *in vivo* [140].

Furthermore, it's possible to use PEGylated dendrimer nanoparticles conjugated binding peptides abundant in glioblastomas. Dendrimers are highly ordered polymeric molecules characterized by a central core and several branching chains developing from the core in a tree-like structure. They are considered very biocompatible, biodegradable, and easily functionalized. Zhao *et al.* developed a small nanoparticle drug delivery system through the conjugation of the fibrin-binding peptide CREKA to PEGylated Polyamidoamine (PAMAM) dendrimer for a potential drug delivery treatment for the clinical therapy of glioma. Fluorescence analysis from *in vivo* and *ex vivo* brain images in U87 orthotopic glioma bearing model mice demonstrated that modified dendrimer NPs could penetrate a deep area of glioma and achieve a significantly higher targeting of glioma site compared to that of the control group and untargeted one. In order to evaluate the penetration of the conjugated NPs in brain tumour tissues, their *in vivo* distribution in glioma slices was imaged, showing a more extended and deeper distribution in the tumour compared to the controls. Furthermore, it was estimated that about 31% of conjugated NPs performed a correct targeting of glioma cells [141].

Other promising affinity molecules are aptamers, which consist of short single-stranded oligonucleotides with high-affinity interactions with 3D structures on the targeted sites. Monaco *et al*. investigated, for example, the possibility to use aptamers, which recognize a specific form of platelet-derived growth factor receptors (PDGFRs) overexpressed in human glial tumours, with polymeric nanoparticles (PLGA-b-PEG) as nanovectors for the targeted delivery of a chemotherapeutic drug. They demonstrated the efficient active targeting and internalization of this nanovector *in vitro* in U87MG GBM cells and the *in vivo* tumour uptake on orthotopic cancer-bearing mice through intravenous administration [142].

Cheng et *al*. incorporated TAT, a BBB-permeable peptide, onto the surface of PEGylated gold NPs with a core diameter of 5 nm. In order to assess the BBB penetrating abilities of gold NPs in vivo, the distribution of particles within the brain of glioma-bearing mice was analyzed 24 hours after intravenous administration of both non-modified and TAT-conjugated gold nanoparticles. It has been shown that both modified and non-modified NPs were able to penetrate the brain microvasculature and reach the brain parenchyma, suggesting their capability to cross the BBB. However, TAT-Au NPs showed a higher level of accumulation within endothelial cells. Furthermore, gold quantification in treated brains demonstrated a ~4.8 fold increase in the percentage of TAT-Au NPs injected dose accumulated in the brain tumour tissue compared to Nm-Au NPs [143].

2.3.3 NPs for Glioblastoma treatment

As mentioned before, NPs physical-chemical properties can be tuned and exploited to direct their functional mode of action. Beside the targeting strategies, nanomaterials for glioblastoma treatment can be classified in two main families: (1) nanocarriers used to encapsulate and deliver anticancer drugs or (2) NPs with intrinsic exploitable physical properties to directly treat the tumour (*i.e.*, thermotherapy and phototherapy).

Among others, size is a crucial factor to consider when designing NPs for glioblastoma treatment. It was shown that dimensions should be optimized according to the tumour stage. During the early stage of progression, smaller nanoparticles (less than 20 nm) are more likely to accumulate at the tumour site, while as the disease progresses, also larger nanoparticles can penetrate the BTB [130].

It is also possible to distinguish nanocarriers for Glioblastoma treatment considering their mechanism of action. Indeed, NPs can be used to encapsulate and deliver anticancer drugs or to

directly treat the tumour exploiting their intrinsic physical properties for thermotherapy and phototherapy.

Nanocarriers for drug delivery of therapeutics

Most of the nanoformulation intended for glioblastoma targeting and treatment are based on PEGylated polymeric NPs. As previously described, hydrophilic polymer surface coatings, such as polyethylene glycol (PEG), are commonly used to increase NPs dispersibility and prolong bloodstream circulation by reducing opsonization. In particular, it was shown that it is possible to determine a PEG density threshold that appears to toggle between fast and slow blood clearance depending on NP diameter [144]. The effect of PEG on NPs fate is also dependent on its molecular weight and polymer chain architecture. PEGylation can be conducted with several techniques using diblock PEG derivatives or by covalently attaching, entrapping, or adsorbing PEG chains onto the surface of a nanoparticle [145].

Technological characteristics and clinical reliability of other polymers have also been extensively investigated, such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) that are highly biocompatible and biodegradable and can encapsulate chemotherapeutics drugs and induce selective toxicity at the glioblastoma target site [146]. For example, Xin *et al.* successfully tested PEG-PCL copolymer nanoparticles in the view of a dual-targeting drug delivery system for Paclitaxel (PTX) delivery [135]. Moreover, Caban-Tokas *et al.* recently proposed a combination therapy of anti-neoplastic PTX or anti-inflammatory R-flurbiprofen separately loaded to PEGylated chitosan-modified PLGA NPs to target RG2 glioma *in vivo*. The uptake of NPs was evaluated with Propidium Iodide-loaded NPs on the RG2 cell line, and it was found to occur rapidly. To test the *in vivo* functionality, NPs were administered intraperitoneally in an RG2 rat glioma model. It was shown that all NPs formulations were able to reach and target the tumour site, reducing its size, and the combination of the two drugs appeared essential for a statistically significant overall effect [147].

NPs made by inorganic materials are of interest for the possibility to exploit the magnetic and optical properties of metals and metal oxide once they reach the tumour site [97,148,149], which will be treated in the following section. Iron oxide nanoparticles (IONPs) have been identified as one of the most promising vectors to increase chemotherapeutic drug delivery [150]. Moreover, since they are efficient carriers for the delivery of iron (Fe^{2+}/Fe^{3+}), they can be useful for performing ferroptosis, recently underlined as a possible target for the treatment of malignant tumours. Ferroptosis is a form of iron-dependent programmed cell death, consisting of the intracellular reaction between excess iron and hydrogen peroxide through the Fenton reaction to generate hydroxyl radicals and singlet oxygen. This leads to a high level of hydroxyl radicals, lipid peroxidation, and eventually to a cytotoxic effect on the cells [151]. Thus, they allow a combined treatment for tumours, including both apoptosis and ferroptosis [152]. Furthermore, IONPs offer several advantages for drug delivery applications since they are biocompatible and biodegradable, and they can interfere with cells' iron metabolism by increasing intracellular iron content. In addition, the superparamagnetic properties make them interesting for imaging purposes. Based on these considerations, Zhang *et al.* designed an IONP-based system to efficiently deliver iron, cisplatin (Pt), and a glutathione peroxidase 4 (GPX4) small interfering RNA (si-GPX4) for the highly efficient synergistic induction of ferroptosis/apoptosis in GBM cells, with iron used as a trigger for the Fenton reaction. Moreover, si-GPX4 was loaded into the nanodrug to maximize the antitumor effect of ferroptosis.. They successfully tested the gene therapy-based IONPs for the treatment of glioblastoma and demonstrated selective U87MG and P3#GBM cells targeting *in vitro* through fluorescence analysis. Moreover, they demonstrated successful *in vitro* and *in vivo* therapeutic effects through the evaluation of tumour growth in immune-deficient nude mice bearing

luciferase-labeled U87MG orthotopic xenografts. [101]. Gold nanoparticles are also good candidates for glioblastoma treatment since they possess many features such as tunable size, a large surface-to-volume ratio, flexibility to surface modifications, and biocompatibility. Cheng *et al.*, as previously saidtested TAT-conjugated gold NPs for the delivery of DXR, a BBB-impermeable drug. Once DXR was bound to NPs, *in vitro* experiments showed that intracellular drug uptake had a 2-fold increase after TAT-Au NPs treatment compared to cells treated with the free drug and *in vivo* experiments proved that the TAT peptide selectively increased the cell uptake of drug at the brain tumour site, thus improving the survival of mice bearing an intracranial U87 glioma [153].

Phototherapy, thermotherapy and magnetic hyperthermia

Some nanocarriers, particularly inorganic ones, possess intrinsic physical properties that can be exploited for treating glioblastoma.

Photothermal therapy (PTT) is a promising non-invasive treatment based on local application of high temperatures to tissues in order to induce irreversible cellular damage at the target site. It involves local irradiation of the tumour thanks to an external near-infrared (NIR) laser, through which the photothermal agent harvests light energy and converts and releases it as heat in a spatially resolved manner. The overall effect of temperature increase causes protein denaturation, cellular membrane disruption, enzyme dysfunction, and mitochondrial corruption, ending up in tumour cell death and necrosis.

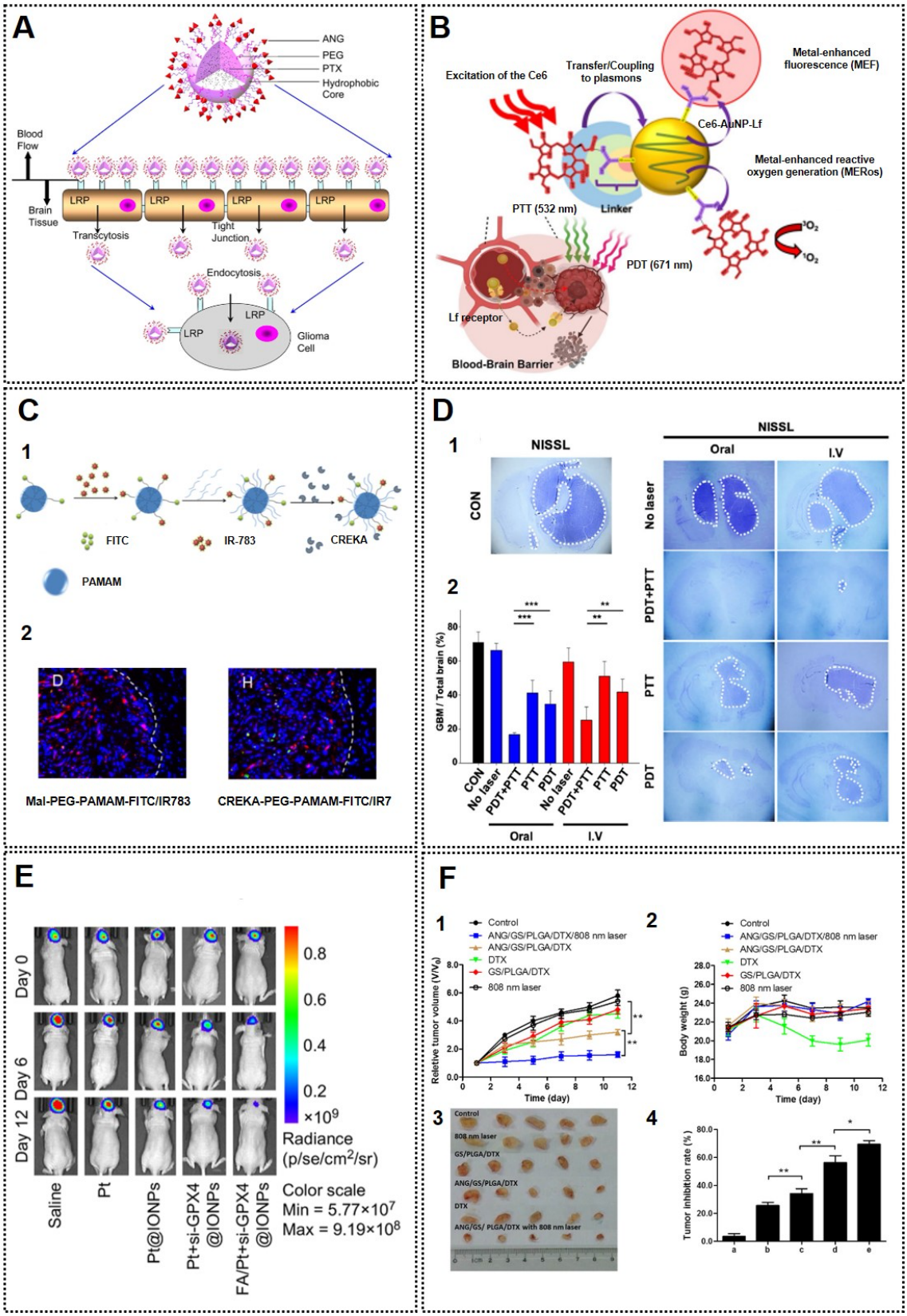
Photodynamic therapy (PDT) also involves the application of light to activate a photosensitizer (PS) that initiates relaxation of its electronically excited state, leading to the production of reactive oxygen species (ROS) and resulting in cell death. The advantage of these techniques as cancer treatment lies in the fact that cancer tissues are more thermosensitive than normal ones, thus allowing maximum effectiveness and limited damage to the surrounding healthy tissues [154]. NPs with intrinsic optical properties, such as gold-based or carbon-based nanomaterials, or loaded with small photosensitizer molecules are excellent candidates for PTT. In this context, Battogtokh *et al.* selected a near-infrared photosensitizer, SiNC, and encapsulated it into a gold nanocage (AuNC). The nanocage was then coated with glycol chitosan (GC) in order to protect the PS from premature release and to improve the biocompatibility of the nanocarrier. They demonstrated that the system applied improved phototoxicity in U87 brain tumour cells compared with free SiNC leading to its significant accumulation in the cancer cells and simultaneous effects of PS and AuNC. The in vivo anticancer efficacy of nanocages in brain GBM-inoculated mice was greater than that of the other groups, suggesting that the NPs were highly localized into tumours [155]. Kim *et al.* developed a system that conjugates glutathione-coated gold nanoparticles (GSH-AuNP) with the photosensitizer Ce6 and with lactoferrin (Lf) via a polyethylene glycol (PEG) linker, where Lf is essential since it is absorbed by interactions with the lactoferrin receptor (LfR) expressed in BBB and GBM. Thanks to this complex system, PDT and PTT could be performed. In particular, the phenomenon that improves the intrinsic ROS generation capacity of Ce6 is mediated by the intersystem crossover between Ce6 and AuNP, and it is referred to as metal enhanced ROS generation (MEROs). They demonstrated that Ce6-AuNP-Lf is capable of crossing both the intestinal and the blood-brain barrier using a human BBB Transwell model and measuring the amount of AuNPs able to cross. Moreover, they confirmed that this ability was due to LfR-mediated transcytosis and the nano size of the system. Furthermore, orally administered NPs showed higher bioavailability and GBM accumulation. Finally, it was assessed that the MEROs-mediated PDT and PTT of the system resulted in significant tumour destruction and growth inhibition *in vivo* in various GBM mice models [156]. Differently, Robinson *et al.* proposed the use of single-layered non-covalently PEGylated reduced graphene oxide (rGO) nanosheets linked to arginine-glycine-aspartic acid (RGD) peptide for GBM PTT. Nano-rGO sheets resulted in high biocompatibility, high NIR light

absorbance, selective cellular uptake in U87 glioma cells, and active photoablation *in vitro*. Moreover, they allowed a combined effect with photothermal and chemotherapy, thanks to the binding with doxorubicin (DOX) [157]. Hao *et al.* developed dual chemotherapy and thermal therapy through a hybrid nanocomposite formed of docetaxel (DTX)-loaded PLGA NP with a discontinuous Au nanoshell bonded to the tumour-targeting peptide angiopep-2 (ANG) in order to achieve active targeting capability. The drug release from the NPSs was triggered by the NIR laser exposure, leading to NP destruction and faster DTX release. It was demonstrated a significant reduction of tumour growth in *in vitro* U87 MG model following systemic administration and laser irradiation [158].

Magnetic iron oxide NPs are also widely applied in cancer thermotherapy induced by a magnetic field (magnetic hyperthermia). In magnetic hyperthermia, IONPs are exposed to an alternating external magnetic field that activates motion of the particles and local heating. This effect can lead to tissue damage in the NPs' surrounding area and can be applied in tumor therapy. In particular, the main effect is based on the increase of the temperature above 42 °C and to the resulting denaturation of proteins and cell death [159,160]. Maier-Hauff *et al.* developed a strategy that involves the direct injection into the tumour of water-dispersed iron oxide NPs that are subsequently heated by an alternating magnetic field. They demonstrated that in a rat model of intracerebral GBM this technique led to an up to 4.5-fold prolongation of survival. They also demonstrated the feasibility and tolerability of thermotherapy using magnetic nanoparticles, alone and in combination with fractionated stereotactic radiotherapy, in a preclinical trial on patients with glioblastoma multiforme [161,162]. Moreover, Hemery et al. investigated the differences between IONPs with two different morphologies (monocore and multicore) in terms of *in vitro* glioblastoma cancerous cell internalization and of the ability of these IONPs to kill cells by magnetic field hyperthermia. The higher efficiency of the multicore IONPs compared to the monocore IONPs was evidenced by an increased cytotoxicity that appeared also to be independent of the iron oxide dose [163]. In another study, Le Fèvre et al.produced biologically synthesized iron oxide nanoparticles, called magnetosomes, that are made biocompatible by removing potentially toxic organic bacterial residues by coating the surface with poly-L-lysine, forming the final system called magnetosomes-poly-L-lysine (M-PLL). They demonstrated M-PLL anti-glioblastoma efficacy, comparing it with that of chemically synthesized iron oxide nanoparticles (IONPs) for magnetic hyperthermia, using a mouse allograft model of murine glioma. Applying a specific magnetic hyperthermia protocol, M-PLL lead to higher antitumor efficacy with full tumor disappearances achieved in 50% of mice compared to 20% for IONPs [164].

Despite the encouraging results in preclinical and few clinical trials, none of these synthetic nanoformulations have been fully translated into clinical applications.

Taking inspiration from natural evolution to overcome the limitations imposed by the molecular interactions at the nanoscale, biomimetic approaches are emerging as an intriguing opportunity to further progress in this field.


A
ANG
PEG
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Blood Flow
Brain Tissue
LRP
Transcytosis
Tight Junction
Endocytosis
Glioma Cell
B
Excitation of the Ce6
Transfer/Coupling to plasmons
Metal-enhanced fluorescence (MEF)
Ce6-AuNP-Lf
Metal-enhanced reactive oxygen generation (MERos)
PTT (532 nm)
Linker
PDT (671 nm)
Lf receptor
Blood-Brain Barrier
C
1
FITC
IR-783
CREKA
PAMAM
2
Mal-PEG-PAMAM-FITC/IR783
CREKA-PEG-PAMAM-FITC/IR7
D
1
NISSL
CON
Oral
I.V
No laser
PDT+PTT
PTT
PDT
2
GBM / Total brain (%)
CON
No laser
PDT+PTT
PTT
PDT
Oral
I.V
E
Day 0
Day 6
Day 12
0.8
0.6
0.4
0.2
×10⁹
Radiance (p/se/cm²/sr)
Color scale
Min = 5.77×10⁷
Max = 9.19×10⁸
Saline
Pt
Pt@lONPs
Pt+si-GPX4 @lONPs
FA/Pt+si-GPX4 @lONPs
F
1
Control
ANG/GS/PLGA/DTX/808 nm laser
ANG/GS/PLGA/DTX
DTX
GS/PLGA/DTX
808 nm laser
Reletive tumor volume (V/V₀)
Time (day)
2
Body weight (g)
Time (day)
3
4
Tumor inhibition rate (%)

Figure 2. (A) *Design of Paclitaxel-loading Angiopep-conjugated polymer nanoparticle as dual targeting drug delivery system for glioblastoma via LRP mediated endocytosis (from Xin et al. 2012)* ***(B)*** *Mechanism of metal-enhanced fluorescence (MEF) and metal-enhanced reactive oxygen generation (MERos) by plasmons coupling between gold nanoparticle and the conjugated-photosensitizer Ce6 and glioblastoma targeting through LfR-mediated pathways of the BBB barrier (adapted from Kim et al. 2022)* ***(C) 1.*** *Scheme of preparation of CREKA-conjugated PAMAM dendrimer nanoparticle for glioblastoma multiforme delivery.* ***2.*** *In vivo distribution of Mal-PEG-PAMAM-FITC/IR783 (left) and CREKA-PEG-PAMAM-FITC/IR783 (right) at 24 h after administration. (Blue: cell nuclei stained by DAPI. Green: FITC-labeled dendrimer nanoparticles. Red: glioma cells expressing red fluorescence protein. White dash lines: border of the glioma) (adapted from Zhao et al. 2015)* ***(D) 1.*** *Nissl staining of mice brain without treatment (CON) or after oral/intravenous administration of Ce6-AuNP-Lf in combination with photothermal and/or photodynamic therapy. White dashed lines indicate remaining GBM.* ***2.*** *Proportion of GBM in the brain without treatment (CON) or after oral/intravenous administration of Ce6-AuNP-Lf in combination with PTT and/or PDT (adapted from Kim et al. 2022)* ***(E)*** *Intracranial tumor growth of the BLI of luciferase-expressing U87MG cells monitored 0, 6, and 12 days using the IVIS-200 imaging system after the in vivo treatments (from Zhang et al. 2020)* ***(F)*** *Anti-glioma efficiency in vivo* ***1.*** *Relative tumor volume of different groups;* ***2.*** *body weight variation of different groups;* ***3.*** *photographs of solid tumors dissected from different groups;* ***4.*** *tumor inhibition rate (a): 808 nm laser (b) GS/PLGA/DTXNP (c) DTX group (d) ANG/GS/PLGA/DTX (e) ANG/GS/PLGA/DTX/808nm laser group; P<0.05 (*), P<0.01(**) (from Hao et al. 2015).*

3. ADVANCED BIOMIMETIC APPROACHES FOR BRAIN TARGETING

As described before, the application of nanomedicine has opened up new treatment options for cancers since NPs can transport high doses of drugs and can be functionalized to specifically target the tumour [165]. This would also reduce the side effects currently associated with radiotherapy and chemotherapy. Thus, nanomedicine is increasingly playing a crucial role in the field of cancer research, both in treatment and in diagnosis. However, very few NPs have been clinically approved to date. One of the main reasons for the failure of the transfer of nanomaterials to the clinic is that NPs fail to adequately overcome biological barriers, which leads to a lower-than-expected accumulation of the drug in the tumour, as well as a nonspecific distribution in the body. In particular, when NPs are administered intravenously, the first obstacle they encounter is the surrounding biological environment with which they interact. In the blood, NPs are exposed to a complex and dynamic system containing thousands of different proteins along with lipids and sugars, known as a biomolecular corona, which can reconfigure their surface characteristics [166–169]. Certain components of the corona, called opsonins, can cause sequestration of the NMs by cells of the mononuclear phagocytic system, resulting in low accumulation in the tumour tissue. Secondly, in the case of GBM, in order to reach the tumour site, NPs must cross the BBB. This barrier is a dynamic structure that functions as a guardian, also reflecting the needs of the brain. It is formed by complex tight junctions between the endothelial cells of the cerebral capillaries, supported by astrocytes and pericytes, and has low endocytic activity (see 2.1.1). As a consequence, the BBB behaves as a continuous lipid bilayer and prevents the passage of polar substances and insoluble lipids. It is, therefore, the main obstacle for drugs that fight diseases that affect the brain [65]. However, the passage through the BBB of different NPs (thanks to their size and chemical-physical characteristics) has been demonstrated, although in a modest amount [170,171]. Finally, once the BBB has been crossed, the NPs directed against GBM might be recognized and eliminated by the neuro-immune system, an additional obstacle to overcome before reaching the cancer cells and

successfully release the therapeutics. The neuroimmune system, composed mainly of glial cells (astrocytes, oligodendrocytes, and microglia) is a system that protects the brain from pathogens, keeping the BBB selectively permeable, mediating neuroinflammation and wound healing, and mobilizing defences against pathogens [171,172]. All these barriers made the fight against GBM, and brain cancers in general, particularly challenging until the intuition of using biological strategies to mimic the nature of our living system. In particular, nanomedicine and NPs evolved toward biomimetic NMs that could be recognized as endogenous from the body cells and tissues, thus increasing their lifetime in the blood circulation and escaping the immune system. Furthermore, the advancements in NP engineering and functionalization allowed the design of incredibly smart nano-tools able to cross the BBB, reach the GBM tumour site and release a specific drug and/or define the cancer boarders for diagnosis. But what does it mean and which characteristics has a biomimetic NP?

3.1 Biomimetic nanoparticles as innovative nanomedicine tool for cancer treatment

The main strategy adopted to avoid protein adsorption and prolong the blood half-life of NPs is the modification of their surface with PEG or with polysaccharides, although recently certain concerns about acquired immunity are emerging in relation to PEG [173]. In the last ten years, a new type of bio-inspired camouflage has gained attention as a novel drug delivery platform: NPs camouflaged by natural cell membranes (nanoghosts - NGs), which have emerged as an innovative type of biomimetic system, endowed with unique functionalities that bridge the gap between synthetic materials and biological entities [174,175]. Several sources of cell membranes (red blood cells, macrophages, mesenchymal stem cells, lymphocytes) have been explored to coat the surface of NPs and prolong their systemic circulation time, highlighting the importance of the cell source on the immunological impact and specific tumour target goal [176,177]. One of the first NGs developed, derived from the mesenchymal stem cell membrane (MSC-NGs) that retain MSC surface markers and behave broadly as MSCs in relation to tumour identification capabilities both *in vitro* and *in vivo* [176]. MSC-NGs have been produced in different sizes and loaded with a variety of therapeutics using a technologically scalable and pharmaceutically applicable process that involves the removal of the cytosol and nuclei residues [178]. The loaded therapeutics can range from small molecule compounds to membrane-bound factors over-expressed in MSCs prior to NG generation and recombinant proteins and DNA constructs. MSC-NG could be in the future a good alternative to MSCs, potentially safer, as they are not associated with the common risks that arise from the administration of living proliferating cells [179]. In this context, Timaner *et al.*, showed that chemotherapy-educated MSCs promote therapy resistance *via* crosstalk with cancer stem cells (CSCs). In response to gemcitabine chemotherapy, MSCs colonized pancreatic adenocarcinomas in large numbers and resided in close proximity to CSCs. In an orthotopic pancreatic cancer model, targeting CSCs using MSC-NGs and loaded with a CXCR3 antagonist enhanced therapy outcome and delayed tumour re-growth when administered in combination with gemcitabine [180]. Furthermore, another successful example of NGs comes from decorating platelets with immune-modulating anti-PDL1 antibodies in order to promote the delivery of anti-PDL1 antibodies to the tumour sites and target circulating tumour cells, and therefore inhibit the tumour recurrence and metastasis. At the same time, the platelets could also target niches for cancer metastasis through recognizing exposed extracellular matrix [181]. Such properties make platelet membranes outstanding platforms for tumour targeting. Moreover, platelet-biomimetic NGs often display elongated blood circulation times and decreased absorption by healthy organs. Jing *et al.*, recently developed a NG by encapsulating melanin NPs (MNPs) and doxorubicin (DOX) inside RGD peptide (c(RGDyC))-

modified nanoscale platelet vesicles (RGD-NPVs) to efficiently inhibit the growth and metastasis of drug-resistant tumours via a cancer cells and tumour vasculature dual-targeting strategy. By taking advantage of the self-recognizing property of the platelet membrane and the conjugated RGD peptides, RGD-NPVs@MNPs/DOX was able to evade immune clearance and target the αvβ3 integrin on tumour vasculature and resistant breast tumour cells [182].

Since intratumoral NP extravasation and penetration are still challenging and represent another obstacle to overcome, cell sources for the camouflage have then been also exploited to improve the accumulation of NPs in the tumour site. In particular, some tumour derived factors induce chemotaxis in monocytes, which are then recruited to the tumour site where they finally can differentiate to mature macrophages by promoting an increased attachment to endothelial cells bordering tumour tissue [183]. Monocytes are then recruited to the tumour site, where they finally can differentiate to mature macrophages. Cell migration is a highly coordinated process requiring dynamic cytoskeletal remodelling among other cellular processes. Accordingly, monocyte membrane-camouflaged NPs were seen to improve the circulation time (by avoiding the immune cell recognition) and the extravasation and accumulation of the NPs in the target tumour [184].

Finally, the tumour microenvironment is another barrier that NPs need to beat in order to target the tumour cells. A hallmark of most solid tumours is the presence of tumour-associated macrophages (TAMs). As a consequence, NPs that come into contact with TAMs will be rapidly recognized. When macrophages show the M2 phenotype, they exert pro-tumour activities, and they correlate with metastasis and poor prognosis. Due to this detrimental role, some strategies have focused on selective targeting of TAMs using NPs in order to deplete and inhibit the recruitment or modulate TAM polarization [185]. Redox and pH unbalance are also others unique characteristics of the tumour microenvironment [186]. They are known to generate a microenvironment favourable to tumour cells, detrimental to the immune response, favouring drug resistance. Classically, high levels of intracellular reactive oxygen species (ROS) are considered carcinogenic. However, growing evidence indicates that tumour development and progression are instead promoted by antioxidants triggered in stressed cells, which prevent ROS-induced cell death and consequently favour the survival of transformed cells [187]. In particular, the xc- antioxidant system promotes tumour growth by protecting against oxidative stress. An additional stress response exploited by neoplastic cells is the membrane expression of proton pumps extruding intracellular protons. In fact, the aerobic glycolysis in cancer cells generates a low pH that would kill them; instead, extruding protons allows cancer cells to survive. Interestingly activated macrophages M2 share with tumour cells both the upregulation of xCT that occurs in response to ROS and the membrane expression of v-ATPases [188]. However, the poor aqueous solubility of some chemotherapeutics and proton pump inhibitors (such as esomeprazole) causes insufficient bioavailability and, thus, limited therapeutic efficiency. In this scenario, lipid-based NPs played a crucial role as promising vehicles for the transport, protection, and delivery of hydrophobic drugs [166,189]. Moreover, they have now also been tested with cell membrane coating in a camouflaged set-up, showing a great versatility in active cancer targeting [190,191].

In summary, cancer cell membrane-based bioengineering approach recently gained much attention and has been consequently applied in the development of NGs possessing homotypic binding and immune escaping functions [176,192]. Unlike ligand-based active targeting, the self-recognition function of biomimetic NPs is primarily dependent on the interaction between natural membrane proteins and target cells, thus avoiding the limitations of receptor expression density variations. Moreover, the immune system has difficulties in recognizing membrane-camouflaged NPs, because they are perceived as source cells with long circulation. Finally, the cancer cell membrane-based biomimetic technology has been applied for molecular imaging, drug delivery, immunotherapy, and theranostics in various cancer models [193]. Based on the homing ability of NGs

to tumours and target cancer cell subpopulations, there is continuous ongoing increasing research in educated-derived NGs as “Trojan horses” for drug delivery. Such NG-based therapy represents a promising strategy to overcome the intrinsic biological resistance barriers against cancers. However, the efficacy of the camouflage of NPs to target CNS tumors, including GBM, has been poorly investigated. Furthermore, in the case of the CNS, the passage through the BBB still remains the main challenge, and, to date, only a few mechanisms have shown a significant increase in transcytosis through the cerebrovascular endothelium (as previously described in 2.2.1) [194,195].

3.3 Nano-biomimetic strategies to target glioblastoma

Targeting brain tumours is not an easy task, mainly due to the great protection role of the BBB that prevents the passage of biomolecules (and nano-cargos) from the bloodstream to the CNS. Moreover, another aspect one needs to take into consideration when designing nanomedicine tools for the brain, is that the presence of many other cell types, such as microglial and astrocytes, may lead to nonspecific uptake and subsequent side effects. As such, it is of utmost importance to explore an effective and targeted drug delivery system able to reach the brain tumours and effectively release the therapeutics, minimizing both invasiveness and unwanted reactions. As briefly discussed in the previous paragraph, NPs coated with biologically derived cellular membranes have recently emerged as innovative and potentially disrupting new class of drug delivery systems that could be used for specific treatment of GBM. However, the use of neural cell-derived membrane coatings remains still undercover and poorly explored. In this scenario, Zhang and colleagues [196] utilized four types of cell membranes derived from the CNS (and in particular from astrocytes, cortical neurons, microglial cells, and oligodendrocyte progenitors) to demonstrate the efficacy of targeted delivery of biomimetics poly(ε-caprolactone) fluorescent NPs. In this work, the authors mainly focused on the design and characterization of the biomimetics NPs by showing a successful coating with all the four types of membranes tested and subsequent extensive screening of these cell membrane-coated NPs on various CNS cells. The cell membranes were obtained by extruding the cells trough a 400 nm membrane filter, subsequently a mixture of cell membrane and NPs were extruded through 200 nm membrane filter and biomimetic NPs were obtained. The results they obtained suggested that microglial and oligodendrocyte progenitors were the most sensitive cell types toward cell membrane-coated NPs. Besides that, another key output of the study was that coating the NPs with four types of the CNS cell membrane reduced the activation of the microglial cell, suggesting high tolerance and biocompatibility of the newly synthesized biomimetics NPs. The study of Zhang and collaborators opens the way to further explore neural-derived cell membranes to fabricate biomimetic NPs able to enter the brain and target specific CNS tumours, and it is indeed a very crucial work for the future of neuro-nano delivery systems. However, the data presented are limited to *in vitro* results, and the authors did not address the BBB crossing limitation step and, more importantly, did not investigate the uptake of NPs by glioblastoma cells.

An alternative biomimetic option is to use proteins as structural building blocks for nanocarriers development directly. Gregory et al. engineered a GBM-targeting synthetic protein nanoparticle comprised of polymerized human serum albumin (HSA) and oligo(ethylene glycol) (OEG), loaded with the cell-penetrating peptide iRGD, which has been shown to increase the accumulation of both small molecule drugs and NP drug delivery systems when delivered or conjugated to NP surface, and STAT3i, involved in signaling pathways related to tumor progression and immune system evasion [197,198]. *In vitro* uptake experiments by GL26 glioma cells showed that iRGD-loaded NPs uptake increased by ~5-fold compared to NPs without iRGD. To evaluate if systemically delivered NPs can reach brain tumours *in vivo*, NPs loaded with Alexa Fluor 647-labelled albumin were

administered via a single tail vein injection in GL26 syngeneic mouse glioma model. Fluorescence imaging demonstrated that a significant number of NPs appeared to have crossed the BBB and to have targeted the tumour compared to iRGD-free NPs. They demonstrated that the incorporation of the tumour-targeting, tissue-penetrating peptide, iRGD, results in an ability of the NPs to penetrate the BBB and distribute throughout the tumur, efficiently delivering siRNA against STAT3, with 87.5% of long-term survival during *in vivo* experiments (reduced levels of STAT3, no residual tumours, normal brain architecture, and no inflammation) [199].

A step forward comes from a very recent study of Zinger and collaborators that used nanovesicles (NVs) as innovative, theranostic tools for cargo delivery [200]. The authors engineered the NV surface with membrane proteins from human neural cells, merging the advantages of designing a biomimetic system for brain targeting with the innovation of adopting human samples. In particular, the optimization and validation of scalable and reproducible production of two types of neuron-targeting NVs, each with a distinct lipid formulation backbone suited to potential therapeutic cargo, was explored by integrating membrane proteins that were unbiasedly sourced from human pluripotent stem-cell-derived neurons. The results showed that both endogenous and genetically engineered cell-derived proteins effectively transfer to NVs without altering their physicochemical properties. Successfully, the NVs with neuron-derived membrane proteins exhibit enhanced neuronal association and uptake compared to bare NVs, and the viability of neural sphere cultures was not disrupted by the treatment, suggesting very high biocompatibility. Although *in vivo* results confirmed cellular association and uptake of the biomimetic humanized NVs to neurons within rodent cranial nerves, the BBB translocation was once again not proved, being the NVs administered directly through intracranial injection. The results displayed are extremely encouraging and leave the debate open whether we will be able to exploit these nanotechnologies in human studies in the near future.

For what concern GBM, one of the first biomimetic approaches used to actively target the tumour site was made in 2019 by Kadiyala and colleagues [201]. The authors exploited the use of a single protein able to form nanostructures and act as a cargo. In particular, HDL-mimicking nanodiscs conjugated to CpG (a TLR9 ligand expressed by several immune cells able to trigger immune rejection) and loaded with docetaxel (DTX), a chemotherapeutic agent, were fabricated and characterized. The idea was to exploit the small size of HDL-nanodiscs to provide accumulation and diffusion through the tumour of the loaded drug. The NPs triggered antitumor CD8+ T cell-mediated immunity and developed a long-term immunological memory *in vivo,* showing encouraging results that prompt researchers to pursue the biomimetic-single protein path to treat GBM. Indeed, another explored system for GBM active targeting was an albumin-based structure for the co-delivery of disulfiram (a copper chelating agent) and the macrophage modulator regorafenib. This system has been used to simultaneously hit glioma cells and protumour M2 macrophages [202]. In this case, the albumin characteristics of targeting SPARC proteins, which are overexpressed on tumour cells and on tumour-associated blood vessels (i.e., on endothelial cells), were exploited to reach cancer cells. Moreover, to render the system more efficient and increase the chances to cross the BBB, transferrin receptor-binding peptide T12 was linked to the NP surface. Overall, the results showed, together with a high tumour specificity, cytotoxic T lymphocyte immune responses and suppression of protumour M2 macrophages.

Among the few strategies explored to design nano-based tools for GBM targeting, the ones adopting lipid-based NPs have been so far the most successful. Specifically, liposomes and lipid-based NPs showed great potential regarding drug encapsulation, high biocompatibility, surface functionalization and BBB crossing [203]. Li *et al.*, in their work, described how biomimetic liposomes modified with transferrin are able to actively target GBM and release elemene (a fat-

soluble small molecular compound that can pass through the BBB and exhibits significant effect on malignant gliomas clinically) and cabazitel (a substance that inhibits proliferation of cancer cells by binding to tubulin) [203]. The liposomes were conjugated with transferrin protein and embedded in RG2 glioma cell membranes (**Figure 3)**. This system was compared to naked classical liposomes and showed high stability, higher significant homologous targeting, and immune evasion *in vitro.* Moreover, the uptake from GBM cells was seen to be much higher compared to control liposomes, also displaying an increased brain drug accumulation and tumour penetration *in vivo*. The active-targeting biomimetic liposomes here studied possess all the characteristics to play a crucial role for the future of GBM treatment, especially since the *in vivo* results showed a higher survival rate and time of mice and lower cytotoxicity compared to the use of the free-drugs commonly used to cure GBM.

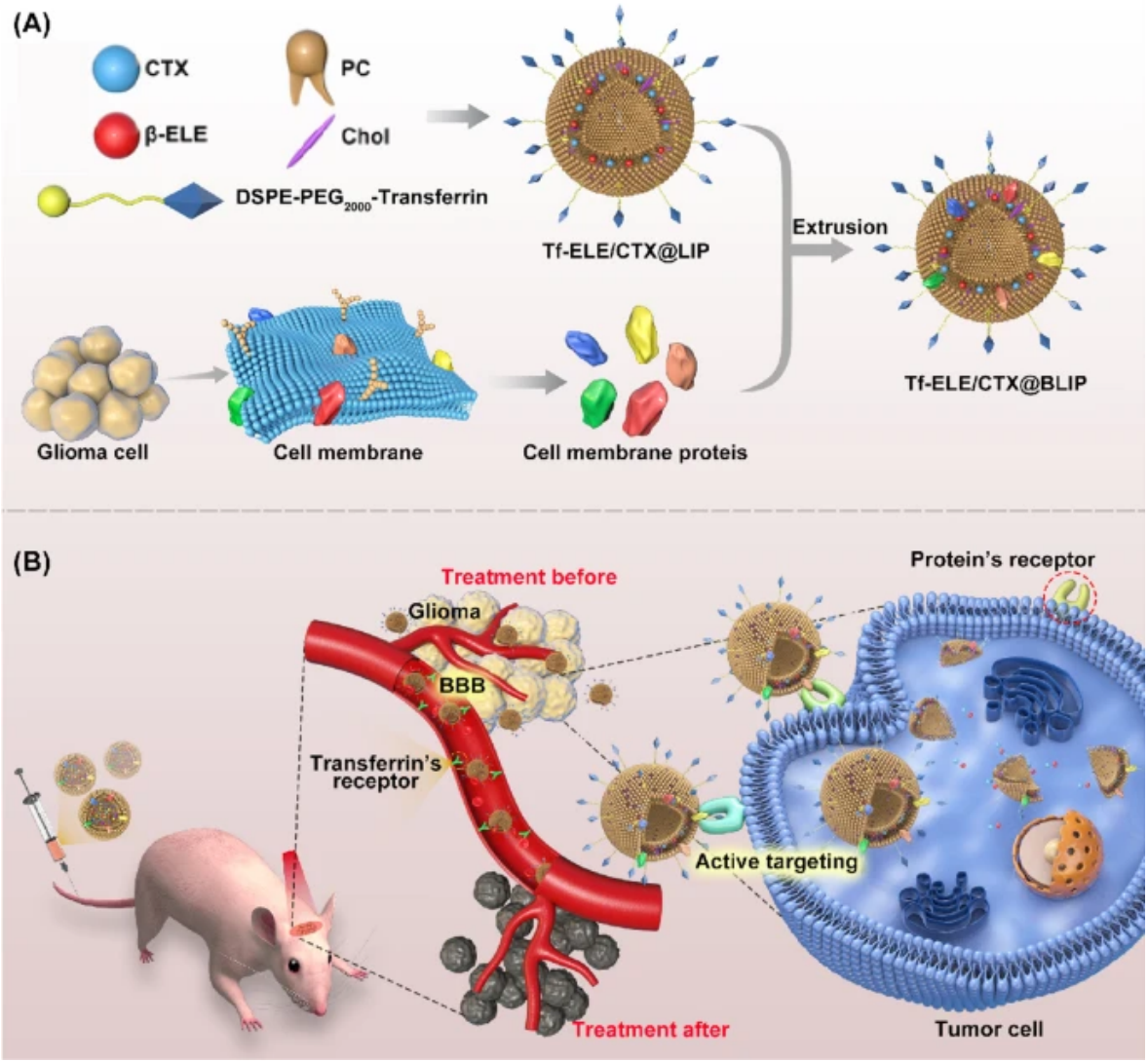

Figure 3. *Schematic illustration of the preparation process of active-targeting biomimetic liposomes engineered with transferrin (Tf-ELE/CTX@LIP) to optimize BBB translocation and homologous targeted delivery in GBM-bearing mice.* ***(A)*** *Preparation process of Tf-ELE/CTX@BLIP.* ***(B)*** *Schematic diagram showing mechanism of penetration of Tf-ELE/CTX@BLIP through the BBB and delivery of chemotherapy to tumor cells. Reproduced with permission from (Li et al, 2021)* [203].

Similarly, Jia and collaborators designed biomimetic proteolipid NPs for active targeting of GBM to be used as an innovative phototheranostic strategy [204]. The authors embedded glioma cell membrane into fluorescent (indocyanine green) proteolipid NPs for fluorescence imaging, tumour margin detection, and phototherapy of orthotopic glioma in mice. Due to the glioma membrane, the NPs were able to cross the BBB and reach the brain, then actively target the GBM due to their excellent homotypic targeting and immune escape characteristics. Once again, a high brain tumour accumulation was achieved. Moreover, the GBM and its margin were clearly visualized by near-infrared fluorescence imaging. As proof of concept, the authors used the imaging guidance to remove the glioma tissue surgically. This point is of extreme importance since one of the most challenging issues of surgical tumour removal is the residual tissue that cannot be extracted from the brain during the procedure. Finally, NIR laser irradiation was adopted as a complemental tool to enhance the therapeutic effect of the NPs. The irradiation produces a photothermal effect that reflects in a very high tumour growth inhibition without affecting the normal brain tissue. This new platform makes an important advance in GBM research therapy by merging both diagnostic (tumour margin detection) and therapy (active targeting of GBM), paving the way toward the future of GBM treatment. Following the lipid-based strategy, Geng and co-authors have developed a biomimetic vehicle based on a low-density lipoprotein (LDL) triggered by Angiopep-2 peptide and ApoB protein [205]. The NPs were loaded with the most used chemotherapeutic agent against GBM, TMZ, and the ApoB protein was seen to facilitate the BBB passage. *In vitro,* the LDL-NPs showed high apoptotic effects towards two classes of GBM, reduced the number of derived endothelial tubules even at low drug concentration, and inhibited endothelial cell migration and angiogenesis. The pharmacokinetics was then studied *in vivo* and displayed a higher brain tissue uptake rate compared to free TMZ, as well as prolonged survival rate of the mice compared to both untreated control group and TMZ only group. The strategy of biomimetic lipid-based NPs showed once again great potential for delivering chemotherapeutic intracranially. Finally, Apolipoprotein E3-reconstitued high-density lipoprotein has been investigated as a nanostructure to encapsulate ATF5 siRNA molecules [206]. This specific siRNA targets the activating transcription factor-5 (ATF5), an overexpressed anti-apoptotic transcription factor in GBM. The Apolipoprotein was seen to facilitate the transport of the siRNA to the GBM, enhancing BBB translocation and actively targeting the tumour cells. The effect of the siRNA increased then GBM cell apoptosis and inhibited tumour cell growth. The results were shown to be extremely promising both *in vitro* and in xenograft cancer model.

Gene therapy, in general, has been introduced as an alternative to radiation and chemical therapy for GBM, and biomimetic NPs could present once again the future for the delivery of such biomolecules. Polyethylenimine (PEI25k)/plasmid DNA (pDNA) complexes have been coated with C6 rat GBM cell membranes [207]. The designed NPs showed great colloidal stability, and their transfection efficiency was seen to be not affected by the presence of serum proteins, suggesting they could maintain a high targeting specificity *in vivo.* The coating with cell membrane improved the biocompatibility of the NPs, compared to naked PEI25k NPs, and increased the homotypic targeting effect as shown by a higher GBM cell uptake. The authors also tested the therapeutic effects *in vivo* in intracranial C6 transplanted GBM animal models by loading the herpes simplex

virus thymidine kinase plasmid (pHSVtk) into the NPs and checking its expression in the brain. The results showed that the biomimetic NPs induced higher HSVtk expression compared with the normal NPs. Furthermore, tumour size was reduced more efficiently by the biomimetic NPs. These results indicate that biomimetic NPs are also suitable and good candidates for gene delivery to the brain, an alternate strategy against the GBM. Moreover, this study suggests that other biomaterials, apart from lipid-based ones, could be used as NP cores for the preparation of biomimetic nano-tools. PolyVinyl Alcohol (PVA) has recently been tested to fabricate biomimetic NPs to treat TMZ-resistant GBM [208]. Here, on top of the material used, two other innovative strategies have been explored: the use of zoledronate (ZOL) as an encapsulated drug, able to induce apoptosis in TMZ-resistant GBM cells, and microglia cell membranes as a coating for the NPs. Such NPs were tested both *in vitro* and *in vivo*, including in a BBB transwell system model, to explore their translocation efficacy through the cerebrovascular endothelium. ZOL-NPs were actively recruited to TMZ-resistant GBM region by CX3CL1/CX3CR1 and CSF-1/CSF-1R signal axis, and the release of the drug was triggered by glutathione in GBM cells. The results showed inhibition of the growth of TMZ-resistant GBM by apoptosis and a decrease in the migration and invasion of TMZ-resistant GBM cells. Besides, by increasing the proportion of M1-type GAM and blocking the expression of HIF-1α, the ZOL-NPs had a significant impact on the immunosuppressive and hypoxic microenvironment. In summary, not only GBM membranes might be used for biomimetic coating but also other CNS cell membranes (as seen at the beginning of this paragraph, [196,200]), including microglia, in order to improve the GBM active targeting by enhancing BBB translocation and improve the immune escaping.

Finally, metal-based NPs have also been explored in the latest two years. Their main advantage is that they can be used to trigger photothermal stimulation, thus specifically destroying cancer cells in the brain. In a first study, Guglielmelli *et al.* present keratin-coated gold (Ker-Au) NPs as highly efficient photosensitive nanosized therapeutics for plasmonic photothermal (PTT) therapy [209]. The authors performed *in vitro* experiments on human GBM cells U87-MG showing efficient cellular uptake and localized photothermal heating capabilities. Moreover, the Ker-Au NPs displayed great biocompatibility. Nevertheless, these kinds of strategies lack drug transport in the tumour site and need to be further explored in order to assess BBB translocation and active targeting of the GBM *in vivo.* It remains anyway a very promising alternative to enhance and improve the PTT therapy already in use for GBM affected patients. Cu2-*x*Se NPs were instead adopted by Wang and colleagues in a very recent study for boosting GBM immunotherapy [210]. The success of emerging immunotherapy in cancer medicine, together with the important side effects that radio- and chemotherapy might present, has attracted considerable interest in the treatment of GBM. However, the unique immunosuppressive microenvironment (TIME) of GBM has led to the failure of immunotherapy, so far. Therein, the authors presented a novel all-in-one biomimetic nano-tool that consists of ultrasmall Cu2-*x*Se NPs with specific intrinsic characteristics, such as photo-responsive Fenton-like catalytic property for inducing immunogenic cell death (ICD) and alleviating the hypoxia of tumour. The NPs have been loaded with Indoximod (an inhibitor of indoleamine-2,3-dioxygenease in tumour), JQ1 (an inhibitor for reducing the expression of PD-L1 by tumour cells), and finally coated with tumour cell membrane for improving the targeting capability. The *in vivo* results in orthotopic GBM models showed a drastic activation of the immune response by increasing M1-phenotype macrophages at tumour site. Moreover, through NIR irradiation, the NPs were able to promote the polarization of tumour-associated macrophages due to ROS generation produced under the Fenton-like reaction between NPs and H_2O_2 present in the tumour microenvironment. The release of the Indoximod decreased the infiltration of Tregs at the tumour site, while JQ1 decreased the expression of PD-L1 on cancer cells. In summary, this approach showed how the modulation of tumour immunosuppressive microenvironment could be

crucial to improve the immunotherapy of GBM, opening the door to new future therapeutical strategies. Of note, the BBB translocation was achieved by temporary opening the tight-junctions of the endothelium using acoustic waves through a 0.5 MHz transducer, and a notable increment of anti-tumour CD8+T cells in the tumour and memory T cells in the spleen was also noticed, suggesting excellent therapy efficacy and effectively prevent the recurrence of GBM.

Ultimately, one last nano-biomimetic strategy worth mentioning that is increasingly gaining attention is based on extracellular vesicles (EVs). EVs are natural biomimetic nanostructures considered extremely good candidates for drug delivery and tumour-targeting since they contain endogenous proteins of their cell type of origin [211,212]. Indeed, the antitumor activity and the tumour-targeting ability of EVs derived from natural killer (NK) cells pre-exposed to IL-15 (able to improve both survival and activation of NK cells), in comparison with EVs isolated from naïve NK cells, has been evaluated by Zhu *et al.* [213]. Of note, the IL-15 derived EVs were demonstrated to express more cytotoxic proteins (such as perforin and FasL) than wild-type NK-EVs. Moreover, their accumulation in the GBM *in vivo* was found to be double of wild-type NK-EVs. The antitumor effect was also improved, although it was not stable after the interruption of the treatment, suggesting that a continued stimulus is required. The same research group also explored the antitumor activity of exosome-mimetic (EMs) vesicles derived from NK cells both *in vitro* and *in vivo* in a xenograft mouse tumour model of GBM [214]. In this work, NK-EMs displayed high cytotoxicity in the various cancer cell lines, including GBM, and they were also able to efficiently cross the BBB and provide tumour targetability and cytotoxicity in a GBM xenograft model.

4. CONCLUSIONS

Malignant GBMs remain an incurable disease due to their intrinsic cellular heterogeneity, their progression phase at the time of diagnosis, and patient variability. Nonetheless, in the last decades, the treatment options have progressively improved and expanded thanks to the better understanding of the biochemical and molecular features of these specific tumors. Immunotherapy, gene therapy, TTFields, and radiolabeled drugs have successfully improved the quality of life and extended the survival rate of patients affected by GBMs, even if the complete eradication of the tumor is at the moment not possible. Unlike other kinds of tumours, in the case of GBM, it is necessary to cross the BBB to reach the tumour site, which represents an additional challenge. This barrier is a dynamic structure that tightly regulates the access of molecules and nutrients into the brain and therefore represents the main obstacle for drugs to fight diseases that affect the brain. Thanks to their peculiar size range, NPs have shown a promising role in carrying substances across the BBB. Although the NPs crossing ability is still modest, many approaches have been developed to improve this capability by decorating NPs with ligands and peptides with high affinity for BBB cellular receptors and transporters. In addition, NPs possess an excellent cargo capability, which makes them attractive for precise drug delivery. Finally, the unique physical properties arising at the nanoscale make functional NPs themselves, subjected to an external wireless stimulus, a weapon against tumours, as in the case of photo/thermo therapy and magnetic hyperthermia.

In the last few years, a plethora of nanotechnology-based strategies to improve active GBM targeted drug delivery have been explored. However, an additional challenge relies in the fact that NPs have to face the immune system (and once overcame the BBB, the neuroimmune system) which often recognize them as external objects. This phenomenon is also exacerbated by the presence of the biomolecules in the blood circulation that spontaneously adsorb onto the NP

surface, forming the so-called biomolecular corona, leading to opsonisation and masking of the targeting moieties.

Therefore, despite the encouraging results obtained with the synthetic nano-approach, an additional step is necessary in view of of a full clinical translatability of these strategies for GBM treatment.

In this view, biomimetics approaches seem to be a valuable tool because of their intrinsic characteristics, including mimicking natural existing cellular structures, thus increasing safety and biocompatibility. As a matter of fact, the side effects of these treatments are remarkably reduced, and there is, in theory, the possibility to create countless variants of such NGs to potentially target not only the GBM but also other brain cancers (by using different CNS cell membranes and load specific therapeutic drugs/biomolecules depending on the cancer cell to be hit). Indeed, one of the main advantages is the facilitate BBB translocation and great homotypic tumour recognition.

What is sure is the future of GBM cure sits on combined treatments, and in this scenario, NPs can serve multiple needs by releasing drugs, enhanced PTT, and imaged tumour margins for diagnosis. Notably, the biomimetic approach highlights the possibility, by exploiting nanomedicine, to design tailor-made treatments for each patient, thus corroborating precision and personalized medicine at the same time.

The major challenge of next years will be to bypass the defects of these novel approaches and eventually to promote the translatability of therapies. In this context, even if there is the possibility to generate very innovative and complex supermolecular NPs, the simplicity and biomimicry in NP design appear to be more successful in the context of scale-up and clinics.

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