Nanomedicines and the Future of Glioma

Dr Aikaterini Lalatsa, MPharm, MPhil, PhD, FHEA
Lecturer in Pharmaceutics, Institute of Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, UK.

Miss Diana Leite, BSc, MSc in Biomedical Engineering
PhD Candidate, Institute of Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, UK.

Professor Geoffrey J. Pilkington BSc PhD CBiol FSB FRCPath
Professor of Cellular and Molecular Neuro-oncology, Institute of Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, UK.

Correspondence to:
Dr Aikaterini Lalatsa,
E: katerina.lalatsa@port.ac.uk

There is a higher incidence of brain tumours in the UK than the world average for both men (8.1 per 100,000), and women (5.3 per 100,000) [1]. Malignant gliomas are the most common primary brain tumours of which glioblastoma (GBM) is the most prevalent. GBM is also known to be the most biologically aggressive and cellularly heterogeneous and is highly diffusively infiltrative in nature which renders surgical excision impossible without causing significant neurological deficit. Typically, following surgery, patients undergo a course of radiotherapy or a combination of chemo/radiotherapy (Stupp protocol) [2]. However, despite surgical debulking and improvements in radio- and chemotherapies, the prognosis of patients with GBM remains extremely poor, with a median survival time of only 14.5 months from diagnosis to death [2].

Particular challenges for GBM therapy are posed by limitations in the extent of feasible surgical resection, chemo- and radio-resistance, difficulties in drug delivery across the blood-brain barrier (BBB, which is intact in tissues surrounding the tumour) and low drug distribution within the tumour and toxic effects on healthy cells. Although it is possible for some cytotoxic drugs to gain access to the major tumour mass by a virtue of damaged or incomplete blood-brain tumour barrier, such drugs fail to reach invading cancer cells which may be centimetres away from perceived edge of the tumour where the BBB is intact.

Nanomedicine, the application of nanotechnology to medicine, works at the molecular level using engineered "bottom up" constructed multifunctional, spatially ordered, architecturally varied nanostructures (Figure 1) to ultimately achieve medical benefit. The field possesses an interdisciplinary conceptual breath bringing together scientists and clinicians towards the fabrication of useful architectures, made up of multiple base parts each with their own structural or functional role driven by discrete molecular forces (chemical bonding, electrostatics, steric interactions and physical adsorption) that will be clinically translated. Within this nanoscale assemblies, specific components are included to tailor the particles properties such as escaping immune recognition, crossing of challenging barriers such as the BBB [3, 4], providing contrast in medical imaging, tumour targeting, stabilising the drug or biomacromolecular therapeutic, controlling its release, and minimising its toxicity (by necessitating for example lower dose and targeting to the desired site). Although active or passively targeted nanoparticulate technologies are the only technologies to-date to have shown promise in delivery across the BBB and in the treatment of GBM, in the field of neurosurgery and specifically glioma therapeutics, there is great interest, and much scepticism, in the rapidly developing application of nanopharmaceuticals [5].

Nanoparticle delivery and glioma targeting

The enhanced permeability and retention (EPR) phenomenon of nanoparticulate accumulation within tumours was first reported in the 1980's while in 1995 nanoparticles were shown to be able to transverse the BBB (Figure 1) [6]. Nanoparticulate delivery systems are tailored

Figure 1: Schematic diagram depicting nanoparticles used in the treatment of GBM (liposomes, polymeric nanoparticles, paptide nanofibers) and targeting nanomedicines to GBM cells.

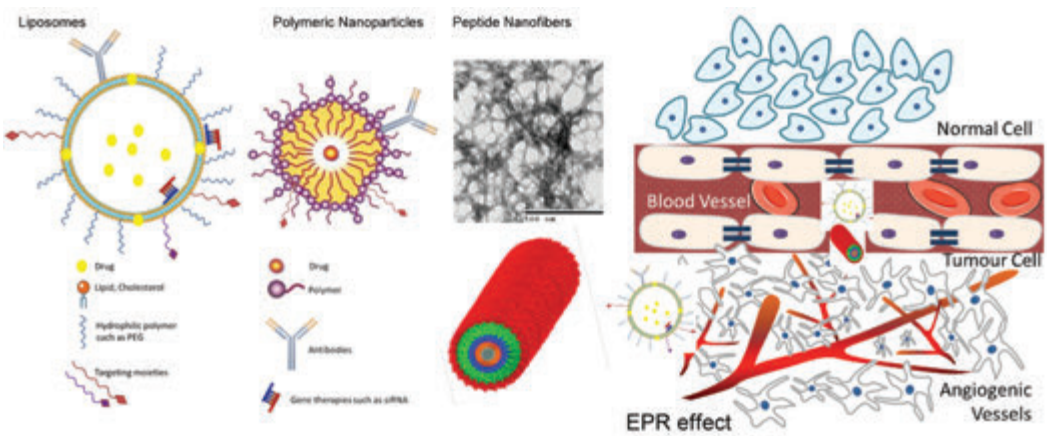

Table 1: Nanoparticle characteristics and their observed impact on tumour localisation					
Size (nm)	**Small**				**Large**
	<10 nm	<20 nm	<70 nm	<100 nm	>150 nm
	Rapid glomerular filtration	Exit tumour cells more easily once internalised (↓EPR)	Improved convective flow through tumour and normal brain	Permits tumour entry via EPR effect	Difficult cell entry via endocytosis – Clearance by RES
Hydrophobicity	Hydrophilic	Amphiphilic	Hydrophobic		
	Increased circulation half-life	Increased BBB permeation	Rapid clearance by RES		
Surface Charge	Cationic	Uncharged	Anionic		
	Adsorptive-mediated BBB trancytosis, cell membrane disruption at high charge	Reduced charge may facilitate spread through tumour ECM	Reduced brain tumour cellular uptake in vivo		
Key; ECM: Extra-cellular matrix, EPR: Enhanced permeation and retention effect, RES: reticuloendothelial system					

in terms of their size, hydrophobicity and surface charge (Table 1) to facilitate tumour targeting and to avoid rapid clearance from the body. Harnessing the EPR effect requires an approximate particle size between 30 -100 nm [6]. Nanoparticles (NPs) with sizes between 15 -100 nm possess a long circulation time compared to smaller particles (10-20 nm) that are rapidly filtered via the kidneys or larger (>150nm) that are uptaken by the reticuloendothelial system (RES) [7-9]. Nanoparticles of optimal size will eventually be uptaken by the liver but they will enjoy a long circulation half-life (2-40 h) [10] which is critical for the accumulation of the nanoparticulate system or the therapeutic across the BBB [4].

The shape of the particles plays a critical role as long axial particles with a diameter of ~20 nm and length larger than 18μm could remain in circulation for longer than 5 days (long enough to mechanically hinder uptake into macrophages) [11]. Long axial particles as peptide nanofibers [3, 4, 12, 13] and carbon nanotubes [14] have shown promise in delivery of therapies such as peptides across an intact BBB enabling 0.4% of the intravenous injected dose to reach the brain. Between the two technologies, peptide nanofibers offer the advantage of low toxicity as are able to be completely enzymatically metabolised to degradation products naturally present within the body and the brain parenchyma as well as high specificity due to their peptide nature [13], and preclinical proof of concept in a murine model [3, 12].

A hydrophobic particle surface is deleterious to a long circulation half-life and coating of particles with low molecular weight surfactants such as polysorbate 80 [15] or hydrophilic polymers notable polyethylene glycol (5 KDa), chitosan or albumin can overcome this problem [16, 17]. Positively charged surfaces promote BBB permeation by physical adsorption to the endothelium with cationic particles readily taken up into the cells at the periphery of tumour spheres compared to anionic [18]. Thus, the potential to maintain a high plasma concentration and interact favourably with the blood-tumour interface make nanoparticles highly useful for glioma targeting.

Table 2: Clinical trials for GBM nanomedicines			
Strategy	**Title**	**Phase/Status**	**ClinicalTrials.gov Identifier**
Liposomes	A study of intraventricular liposomal encapsulated Ara-C (DepoCyt) in patients with recurrent GBM	Phase 1/Terminated	NCT01044966
	Liposomal doxorubicin in treating children with refractory solid tumours	Phase I/Completed	NCT00019630
	Pegylated liposomal doxorubicin and prolonged temozolomide in addition to radiotherapy in newly diagnosed GBM	Phase I and II/Completed	NCT00944801
	Maximum tolerated dose, safety, and efficacy of rhenium nanoliposomes in recurrent GBM	Phase I and II/Not yet recruiting	NCT01906385
	A phase I trial of nanoliposomal CPT-11 (NL CPT-11) in patients with recurrent high-grade gliomas	Phase I/Completed	NCT00734682
	Study of convection-enhanced, image-assisted delivery of liposomal-irinotecan in recurrent high grade glioma	Phase I/Enrolling by invitation	NCT02022644
	An open-label, phase I/IIa, dose escalating study of 2B3-101 in patients with solid tumours and brain metastases or recurrent malignant glioma.	Phase I and II/Active, not recruiting	NCT01386580

Table 3: Preclinical studies of nanomericines for glioma			
Nanoparticles	Drug/Targeting moiety	Outcomes	Ref
Passive Transport			
Lipid Nanocapsules	Curcumin	Seven days after implantation, rats bearing C6 orthotopic tumours were treated with lipid curcumin loaded nanocapsule (1.5mg/kg/day IP, 14 days). A decrease in tumour size and a prolonged animal survival (39 days) compared to saline-treated animals (30 days) was found.	[21]
Polysorbate 80 coated PBCA Nanoparticles	Temozolomide (TMZ) or Doxorubicin/[Low density lipoprotein (LDL)]	Higher concentrations observed in the liver, spleen, and lungs when TMZ was bound with nanoparticles. In the brain, compared with TMZ solution, overcoated nanoparticles significantly increased the accumulation of the drug by 2.29-fold (1.10 ± 0.19 μg/g versus 0.48 ± 0.11 μg/g, IV dose 10mg/kg Temozolomide). Increase survival in 101/8 rat models after IV admistration at 2,5,8 days after implantation of doxorubicin overcoated nanoparticles (2.5mg/kg, 35 days) compared to saline, doxorubicin alone, doxorobucin PBCA nanoparticles (24.5, 27, 35 days respectively).	[22]
Adsorptive Endocytosis			
Wheat germ agglutinin (WGA) - and tamoxifen-coupled Liposomes	Daunorubicin and quinacrine (Tamoxifen inhibits ABC transporters)	In vitro, multifunctional liposomes (uncharged, ~100 nm) were able to permeate across the BBB via adsorptive endocytosis. In a GSC ICR murine model, the survival ranges of mice treated with the saline, daunorubicin and quinacrine liposomes or WGA-tamoxifen-liposomes was enhanced from 26, 30.83 to 36.33 days respectively when a dose of 5mg/kg was injected IV on days 10, 12, 14, and 12 post tumour implantation.	[20]
Active Transport – Lipidic Nanomedicines			
Cationic Liposomes	Doxorubicin/Lactoferrin	Rats bearing C6 orthotopic tumours were injected IV with 3 doses of 2.5 mg/kg on days 3, 6, and 12 post tumour implantation. Treatment resulted in prolongation of survival between lactoferrin-targeted liposomes (96.13 days) and doxorubicin liposomes (56.87 liposomes).	[23]
T7- and TAT- Liposomes	Doxorubicin/Transferrin	BALB/c bearing C6 orthotopic spheroid tumours injected IV with 3 doses of 2.5 mg/kg on days 8, 11, and 14 post tumour implantation showed an enhanced median survival time (43 days) compared to saline group (17 days).	[24]
TAT-cholesterol-conjugated Liposomes	Doxorubicin/Transferrin	Rats bearing C6 orthotopic tumours were injected IV with 3 doses of 2.5 mg/kg on days 3, 6, and 12 post tumour implantation. Treatment resulted in prolongation of survival between lactoferrin-targeted liposomes (79.4 days) and doxorubicin liposomes (57.50 liposomes).	[25]
p-aminophenyl-α-D-manno-pyranoside and Tf-coupled Liposomes	Daunarobucin/ Transferrin and GLUT-1	Increased transport ratio up to 24.9% across in vitro BBB model. Rats bearing C6 orthotopic tumours were injected IV with 3 doses of 5 mg/kg on days 8, 10, and 12 post tumour implantation. Median survival time of tumour bearing rats (22 days) was longer than saline 13 days) and daunorucin-liposomes (18 days).	[26]
Tf- and tamoxifen-coupled Liposomes	Epirubicin (Tamoxifen inhibits ABC transporters)/Transferrin	Rats bearing C6 orthotopic tumours were injected IV with 3 doses of 5 mg/kg on days 7, 9, and 11 post tumour implantation. Treatment resulted in a significant reduction in tumour volume and prolongation of survival between Tf-tamoxifen liposomes (23 days), epirubicin liposomes (17 days), epirubicin alone (15 days) and saline (12 days).	[27]
Octa-arginine (R8) and RGD-coupled Liposomes	Paclitaxel/αvβ3 integrin	BALB/c mice were IV injected with saline, free PTX, PTX-PEG-liposomes, PTX-R8-RGD-liposomes, PTX-R8-liposomes and PTX-RGD-liposomes (3 mg/kg) at 4, 6, 8, 10, 12 and 14 days after implantation and enhanced survival was observed in PTX-R8-RGD-liposomes 26,32,39,48,36,38 respectively).	[28]
p-aminophenyl-α-D-mannopyranoside-D-α-tocopheryl- and dequalinium-coupled Liposomes	Paclitaxel and artemether/GLUT-1 (& adsorptive endocytosis)	Induction of apoptosis in brain cancer cells and brain cancer stem cells by activating apoptotic enzymes and pro-apoptotic proteins and inhibiting anti-apoptotic proteins. The median survival time of rats bearing C6 orthotopic tumours treated with the functional targeting paclitaxel plus artemether liposomes (35 days) was significantly longer than that of rats treated with physiological saline (17 days), taxol (22 days), paclitaxel liposomes (24 days), paclitaxel plus artemether liposomes (25 days), MAN-targeting paclitaxel plus artemether liposomes (28 days), and DQA-mediated targeting paclitaxel plus artemether liposomes (29 days), respectively.	[29]
Angiopep-2-cationic Liposomes	Paclitaxel and pEGFP-hTRAIL gene /LDL	Targeting delivery system improved uptake and gene expression not only in U87 MG cells. Median survival time of U87MG- tumour-bearing BALB/c mice treated with liposomes was 69.5 days, significantly longer than other groups (50 μg pEGFP-hTRAIL and 5 μg PTX per mouse), even longer than the TMZ positive control group (47 days, 50mg/kg) (animals dosed at 7,9,11,13 days).	[30]

Angiopep-2 and neuropilin-1-coupled Liposomes	Docetaxel (DTX) and VEGF siRNA/LDL & Neuropilin-1 receptor	The dual peptide-modified liposomes showed superiority in anti-tumour efficacy, combination of anti-angiogenesis by VEGF siRNA and apoptosis effects by DTX, after both intratumour and system application against mice with U87 MG tumours, and the treatment did not activate system-associated toxicity or the innate immune response.	[31]
Anti-EGFR-coupled Liposomes	Sodium borocaptate/ EGFR	In an animal model of glioma, both liposomes and sodium borocaptate were only observed in the tumour. The therapeutic effect was confirmed by inductively coupled plasma-atomic emission spectrometry both in vitro and in vivo.	[32]
Active Transport – Polymeric Nanomedicines			
Tf-and-cyclo-[Arg-Gly-Asp-dPhe-Lys] (c[RGDFK]) – paclitaxel conjugated Micelle (TRPM)	Paclitaxel (PTX)/ Transferrin	TRPM enhanced mean survival time of mice bearing intracranial U87 MG glioma treated with TRPM (42.8 days) than those treated with Tf modified PTX loaded micelle (39.5 days), PTX loaded micelle (34.8 days), Taxol© (33.6 days), and saline (34.5 days)	[33]
Angiopep-coupled poly(ethylene glycol)-poly(caprolactone) Nanoparticles	Paclitaxel (PTX)/LDL	Enhanced accumulation of ANG-NP in the glioma bed and infiltrating margin of intracranial U87 MG glioma tumour-bearing in vivo model were observed by real time fluorescence image.	[34]
Aptamer (AP) AS1411-coupled poly(ethylene glycol)-poly(lactic-co-glycolide) Nanoparticles	Paclitaxel (PTX)/ Nucleolin	Prolonged circulation and enhanced drug accumulation at the tumour site in vivo. Prolonged circulation and enhanced PTX accumulation at the tumour site was achieved for Ap-PTX-NP, resulting in prolonged animal survival on rats bearing intracranial C6 gliomas when compared with PTX-NP and Taxol® (3 mg/kg, every 2 days for seven consecutive injections until the 20th day).	[35]
APTEDB-conjugated poly(ethylene glycol)-poly(lactic acid) Nanoparticles	– /Extra-domain of tumour-associated fibronectinfibronectin extra domain B (EDB)	APTEDB-NP-PTX exhibited improved anti-glioma efficacy over unmodified nanoparticles and Taxol® in both subcutaneous and intracranial U87MG xenograft models and enhanced the medium survival time of the mice treated with saline, Taxol®, NP-PTX and APT-NP-PTX was 19, 24, 31, 41 days, respectively.	[36]
tLYP-1-functionalised poly(ethylene glycol)-poly(lactic acid) Nanoparticles	Paclitaxel/Neuropilin	Survival of BALB/c mice bearing intracranial U87MG glioma was enhanced (Mice treated with saline, Taxol®, NP-PTX and tLyp-1-NP-PTX survived for 18, 23, 28, 37 days respectively). Dose was set at 5mg/kg every 3 days over 2 weeks.	[37]
F3-functionalised poly(ethylene glycol)-poly(lactic-co-glycolide acid) Nanoparticles	Paclitaxel/ Nucleolin	Survival after IV administration of four groups (saline, NP, F3-NP were injected into mice bearing intracranial C6 glioma at the dose of PTX 5 mg/kg, and the co-adminstration peptide tLyp-1 were given at the dose of 4 μM/kg 5 min after the NPs injection). Following co-administration with tLyp-1 peptide, F3-nanoparticles displayed enhanced accumulation at the tumour site and promoted longest survival in mice [Saline: 9 days, Taxol: 24 days, NP: 27 days, F3-NP: 32 days, Np and tLYP-1: 31 days, F3-NP and tLyp-1: 42 days)]	[38]
iNGR-conjugated poly(ethylene glycol)-poly(D,L-lactic-co-glycolic acid) Nanoparticles	Paclitaxel/ Neuropilin-1 and Aminopeptidase N	iNGR-nanoparticles exhibits significantly enhanced cellular uptake in human umbilical vein endothelial cells, improves the anti-proliferation and anti-tube formation abilities of paclitaxel in vitro. In vivo, it was verified an improved anti-angiogenesis activity and significantly prolonged survival time in mice bearing intracranial glioma (42.5 days).	[39]
MT1-AF7p-coupled-poly(ethylene glycol)-poly(D,L-lactic-co-glycolic acid) Nanoparticles	Paclitaxel/ Membrane type-1 matrix metalloproteinase	The median survival of mice bearing C6 glioma treated with MT1-NP-PTX and iRGD (60 days) was significantly longer than those of mice treated with physiological saline, Taxol®, NP-PTX, NP-PTX and iRGD, MT1-NP-PTX (21, 24,32, 40, 48 days, respectively) [PTX dose 5 mg/kg, iRGD dose 4 ▫mol/kg, animals dosed at day 7, 10, 13, 16, 19 and 22 post implantation].	[40]

Key: APT: Aptamer peptide or aptide, APTEDB: aptide specific for extra-domain of tumour-associated fibronectin B, BBB: blood-brain-barrier, EGFR: epidermal growth factor receptor, F3 peptide: Cys-Lys-Asp-Glu-Pro-Gln-Arg-Arg-Ser-Ala-Arg-Lys-Ser-Ala-Lys-Pro-Ala-Pro-Pro-Lys-Pro-Glu-Pro-Lys-Pro-Lys-Lys.Ala-Pro-Ala-Lys-Lys, G-22-MAb: anti-glioma monoclonal antibody, iNGR peptide (Cys-Arg-Asn-Gly-Asn-Gly-Pro-Asp-Cys), MT1-AF7p : Phase display peptide (His-Trp-Lys-His-Lys-His-Asn-Thr-Lys-Thr-Phe-Leu) with high specificity to MT1-MMP (Membrane type-1 matrix metalloproteinase), Neuropilin: a modular transmembrane protein identified as a receptor for various forms and isoforms of VEGF, pEGFP-hTRAIL: human tumour necrosis factor-related apoptosis-inducing ligand, PTX: Paclitaxel, RGD: Arg-Gly-Asp, GLUT-1: glucose transporter-1,T7: His-Ala-Ile-Ty-Pro-Arg-His, siRNA: small interfering RNA, NP: nanoparticles, TAT: cell penetrating peptide (Ala-Tyr-Gly-Arg-Lys-Lys-Tyr-Tyr-Gln-Tyr-Tyr-Tyr), Tf: transferrin, tLyp-1: a truncated form of Lyp-1 (Cys-Gly-Asn-Lys-Arg-Thr-Arg), TMZ: Temozolomide, VEGF: Vascular endothelial growth factor.

In a preclinical phase, the most significant survival benefits in orthotopic animal models have been achieved with lipidic or other polymeric nanomedines (Table 3) even if a wide variety of particles (dendrimers, peptide nanofibers, carbon nanotubes, and solid lipid nanoparticles) are under investigation.

Liposomal Nanomedicines

Only liposomal particles (spherical vesicles composed of a lamellar phase lipid bilayer) are undergoing clinical trials for GBM, but the outcomes of these studies are still awaited (Table 2). The faster progression of liposomal formulations of chemotherapies to the clinic can be attributed to the fabrication of liposomes using GRAS excipients and that liposomal nanomedicines loaded with doxorubicin, vincristine, daunorubicin, or cytarabine (Depocyt®) for the treatment of systemic cancers have been approved by the FDA (Doxil® for Kaposi's sarcoma approved in 1995, Caelyx® for metastatic breast cancer approved by EMA in 1996 and Lipodox® pegylated doxorubicin liposomes for metastatic ovarian cancer approved by the FDA in 2013) [19]. Although, currently several intravenous nano-enabled chemotherapies are approved for various cancer indications, so far none is approved for a brain tumour indication. Depocyt® underwent phase I and II trials for central nervous system metastases from melanoma and breast cancer (Table 2), however, the results of this study are not yet available.

Drugs can be loaded to the particle surface, intercalated within the lipidic bilayer and also within the core of liposomes. For adequate brain delivery, liposomal formulations need to be pegylated (PEG chain length 2-5 kDa in length) to confer adequate steric hindrance and stabilisation of the liposome, mask the surface charge and reduce opsonisation enhancing their circulation half-life. Similarly, delivery of liposomes loaded with chemotherapies for delivery to the central nervous system necessitates a cationic charge conferred by the fabrication of liposomes using cationic phospholipids such as 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) via adsorptive endocytosis [20]. However, if this pathway is not utilised, liposomes are able to permeate the BBB only when decorated with a ligand for a receptor expressed on the BBB to enable the endocytosis of the particle (Table 3). In some cases, dual functionalisation may also be required to enable targeting of the liposomes to the glioma such as transferrin, insulin, folic acid, neuropilin and epidermal growth receptor. (Table 3).

Polymeric Nanomedicines

Passive transport however, has been achieved mainly only for polymeric nanoparticles loaded with drugs on the surface or core of the particle (Table 3). Drug loading is in majority of the cases higher in polymeric nanoparticles compared to lipidic, for hydrophobic poorly soluble drugs, as well as for hydrophilic biomacromolecules and gene therapies. These carriers also show a higher stability including stability upon dilution in biological fluids and against enzymatic metabolism.

While polymeric nanoparticles are typically thought of as passive smart delivery vehicles, they can also be engineered to actively target tumours targeting a number of receptors either to gain entry across the BBB or overexpressed on GBM cells with similar chemistries and with enhanced possibilities to those relevant to lipidic nanoparticles. There has been an increased number of successful animal studies in mice and rats over the last decade resulting in an enhanced median survival increase of ideally ~20 days [41]. Care must be taken in comparing the results of these studies, however, as different animal models have been used and only a few studies were complemented by pharmacokinetic studies. Xenografts derived from neurosphere cultures and from biopsy spheroid cultures as well as several genetically engineered mouse models more faithfully reflect the genotypic and phenotypic changes seen in human GBMs and recapitulate the infiltrative growth of human gliomas [42], compared to xenografts from chemically induced models as well as normal glioma cell lines grown in serum-supplemented media. Delays in translation of polymeric nanoparticles into the clinic arise from the need of rigorous testing of short- and long-term particle safety (including immunogenicity) and more detailed study of particle biodistribution in large animal models [43] as well as development of protocols permeating the scale-up of the production of loaded and functionalised nanoparticles maintaining target molecule attachment under conditions of clinical-grade sterility.

Toward a cure – Nanotechnology in the operating room and in the clinic

Nano-enabled platforms, rather than simply allowing treatment through a single modality, offer the realistic opportunity for multi-modal treatment, employing many useful approaches simultaneously. Thus, a number of therapeutic strategies can be incorporated via a common nanoscale agent for targeted delivery or even theranostic applications. Thus, a hypothetical particle can be injected intraoperatively after resection carrying iron oxide for example for identification of the margins of the tumour bed under a magnetic field [44, 45], while also carrying one or more targeting agents to promote internalisation past the BBB and to the tumour cell nucleus if needed. After surgery, nanoparticle enhanced ionising-beam therapy could be chosen acting synergistically with the chemotherapeutic drugs loaded or conjugated within the particle. If resection is not indicated, due to the deep intracerebral localisation of the tumour, CED (convection enhanced delivery) of the nanoparticulate formulation possibly taking place at the same time as stereotactic biopsy can be an option. The latter would be followed by imaging to ensure absence of off-target diffusion (e.g. to the brainstem or near large cerebral vasculature). Molecular engineering allows the choice of simultaneous treatment strategies acting synergistically towards tumour cell eradication at the edge of the tumour and beyond. Such combined therapeutic strategies will likely be translated for clinical testing within the next decade based on existing clinical and preclinical trials. Thus, rather than a substitute for surgical therapy, nanomedicines will provide an adjunct to modern surgical strategies, improving the extent of resection, working non-invasively towards tumour eradication of remaining tumour cells and enable the targeting of biomolecular mechanisms that make GBM challenging to treat.

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