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Drug and cell encapsulation: Alternative delivery options for the treatment of malignant brain tumors ☆

Swapnil V. Bhujbal [a,b,*], Paul de Vos [b], Simone P. Niclou [a]

[a] NorLux Neuro-Oncology Laboratory, Department of Oncology, Centre de Recherche Public de la Santé (CRP-Santé), 84, Val Fleuri, L-1586 Luxembourg, Luxembourg
[b] Department of Pathology and Medical Biology, Immunoendocrinology, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands

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ABSTRACT

Malignant brain tumors including glioblastoma are incurable cancers. Over the last years a number of promising novel treatment approaches have been investigated including the application of inhibitors of receptor tyrosine kinases and downstream targets, immune-based therapies and anti-angiogenic agents. Unfortunately so far the major clinical trials in glioblastoma patients did not deliver clear clinical benefits. Systemic brain tumor therapy is seriously hampered by poor drug delivery to the brain. Although in glioblastoma, the blood brain barrier is disrupted in the tumor core, the major part of the tumor is largely protected by an intact blood brain barrier. Active cytotoxic compounds encapsulated into liposomes, micelles, and nanoparticles constitute novel treatment options because they can be designed to facilitate entry into the brain parenchyma. In the case of biological therapeutics, encapsulation of therapeutic cells and their implantation into the surgical cavity represents another promising approach. This technology provides long term release of the active compound at the tumor site and reduces side effects associated with systemic delivery. The proof of principle of encapsulated cell factories has been successfully demonstrated in experimental animal models and should pave the way for clinical application. Here we review the challenges associated with the treatment of brain tumors and the different encapsulation options available for drugs and living cells, with an emphasis on alginate based cell encapsulation technology.

Contents

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* Corresponding author at: CRP-Santé, NorluxNeuro-Oncology Laboratory, 84, Val Fleuri, L-1586 Luxembourg, Luxembourg. Tel.: +352 26 970 273; fax: +352 26 970 390.
E-mail addresses: swapnil.bhujbal@crp-sante.lu (S.V. Bhujbal), p.de.vos@umcg.nl (P. de Vos), simone.niclou@crp-sante.lu (S.P. Niclou).

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1. Introduction to brain tumors

Malignant brain cancer is a devastating disease and associated with very poor prognosis [1]. With an incidence of about 10 in 100,000 people, brain cancer is considered a rare disease but the mortality is very high with half of the patients presenting an incurable tumor type [1]. Pediatric brain tumors are the second leading cause of cancer-related deaths in children under the age of twenty [2]. Tumors of the central nervous system (CNS) are classified based on the presumed tissue of origin, i.e. tumors of neuroepithelial origin, tumors of cranial and paraspinal nerves, tumors of the meninges, lymphomas and hematopoietic neoplasms, germ cell tumors, tumor of the sellar region and metastatic brain tumors [3]. The majority of malignant brain tumors in adults are of neuroepithelial origin and belong to the group of gliomas, based on their resemblance to glial support cells of the brain, astrocytes and oligodendrocytes. Glial tumors are further classified in grades (I to IV) according to their clinical manifestation and malignancy. Except for grade I pilocytic astrocytomas, all other glial tumors eventually develop into a fatal tumor albeit with different incubation times. All these tumors are thus considered malignant. Diffusely infiltrating gliomas (grade II) mostly affect young adults with a high degree of cellular differentiation and slow growth. Over time these tumors evolve to anaplastic astrocytomas or oligodendrogliomas (grade III) or to glioblastomas (GBM). Grade IV astrocytoma or GBM represents the most malignant type of brain tumor in adults and is also the most frequently occurring primary brain tumor. Despite an aggressive treatment regimen, the median time from diagnosis to death for GBM patients is only 14 months [4]. Histopathological features include nuclear atypia, high cellularity, cellular pleomorphism and high mitotic activity. Prominent microvascular proliferation and/or necrosis represent essential diagnostic features. By magnetic resonance imaging (MRI), GBMs display areas of contrast enhancement indicating a disrupted blood brain barrier and neovascularization. Although most GBMs appear de novo as primary GBMs, some evolve from lower grade astrocytomas as secondary GBMs. Due to their strong infiltrative capacity they cannot be effectively removed by neurosurgical resection, and recurrence is inevitable. Invading cells can reside several centimeters outside the contrast enhancing rim and even reach the contralateral hemisphere.

In recent years extensive molecular characterization of gliomas using next generation sequencing, gene expression, copy number alterations and DNA methylation analysis has allowed an improved subgrouping based on genetic features [5–7]. This has e.g. led to the identification of a novel mutation in a gene coding for isocitrate dehydrogenase (IDH) in a subgroup of GBM samples [6]. Later it was found that mutations in IDH1 or IDH2 appear early in the disease course and are characteristic of grade II and III gliomas and secondary GBMs. More than 80% of these tumors carry the mutation, while less than 5% of primary GBMs do so [8,9]. Thus primary and secondary GBMs appear to be different biological entities, although histopathologically they are indistinguishable. In primary GBMs three important signaling pathways are consistently altered, these include receptor tyrosine kinase (RTK) signaling leading to increased cell proliferation, the p53 pathway involved in cell survival and metabolism, and the retinoblastoma (Rb) pathway regulating cell cycle activity [5]. The majority of primary GBMs display an amplification of the epidermal growth factor receptor (EGFR) gene, and additionally often express a truncated version of the receptor (EGFR variant III) which is constitutively active and is associated with increased aggressiveness [10]. These studies also highlighted the remarkable degree of genetic heterogeneity between GBMs and the close correlation between molecular markers and patient outcome.

In addition to primary brain tumors, metastatic brain tumors represent a major clinical challenge since they always constitute a fatal disease progression. Brain metastases are 2–3 times more frequent than primary brain tumors and like these are notoriously difficult to treat because the systemically delivered drugs affecting the primary tumor often do not reach the metastatic sites in the brain. Particularly lung, breast, colorectal cancer and melanoma have a tendency to metastasize to the brain.

2. Opportunities and challenges in brain tumor treatment

At present the standard treatment of GBMs is multimodal and includes surgical resection followed by radiation therapy (RT) and temozolomide (TMZ) based chemotherapy [4]. Despite this intensive treatment regimen the five year survival rate of GBM patients is below 10% [11]. Many alternative therapies are actively being tested that go beyond unspecific cytotoxic agents and aim towards a more tumor specific approach. These include targeted molecular therapies with RTK inhibitors, immune-based therapies and anti-angiogenic treatments [12,13].

Unfortunately small molecule inhibitors targeting RTKs, including the EGFR inhibitors erlotinib, gefitinib and lapatinib, have shown limited efficacy in GBM patients, although pre-clinical studies often produced promising results. Promising downstream targets of RTK signaling involve mTor, protein kinase C, Akt and PI3 kinase [13]. Currently antibodies against EGFR and vaccine strategies including EGFR and EGFRvIII are being explored. Additional promising avenues involving the immune response are dendritic cell, T cell and natural killer cell-based therapies [14]. A recent successful strategy in mice using intracerebral injection of EGFRvIII-specific chimeric antigen receptor transduced T cells holds promise for application in patients with EGFRvIII-expressing brain tumors [15].

As aberrant angiogenesis represents a major pathological feature in GBMs, multiple therapeutic strategies have been developed to target this process. Hope was put into bevavizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), the major pro-angiogenic molecule produced by GBM. Although initial small scale clinical trials indicated a strong increase in progression free survival [16–18], the clinical benefit from this remained unclear, since progression is based on imaging parameters directly affected by anti-angiogenic agents which interfere with adequate quantification of tumor growth [19]. Two recent phase 3 clinical trials comparing bevacizumab to standard of care treatment in newly diagnosed GBM (AVAglio and RTOG 0825) unfortunately did not report any positive effect on overall patient survival [20,21]. Although it remains to be seen whether a subpopulation of patients may benefit from bevacizumab treatment, it is unlikely that bevacizumab will play a major role in the management of GBM. Nevertheless combination therapies with anti-angiogenic agents remain possible. In order to counteract the metabolic adaptation of tumor cells under hypoxia, the combined targeting of angiogenesis and metabolic pathways remains an interesting avenue that awaits further exploration [7].

3. Circumventing the blood brain barrier in brain tumor treatment

In addition to the low efficacy of current drugs, drug delivery from the circulation to the brain is seriously hampered by the blood brain barrier (BBB). The BBB is composed of specialized brain endothelial cells, pericytes and astrocytic endfeet and strictly regulates the passage of large and small molecules between the blood and the brain parenchyma [22]. This structure is essential to protect the healthy brain from blood derived noxious factors, but strongly impairs drug delivery in the diseased brain. Several pre-clinical studies have convincingly shown that the inefficacy of many clinical trials for brain tumors may be partially explained by limited drug availability at the tumor site. E.g. systemic administration of monoclonal antibodies to EGFRvIII led to tumor shrinkage in subcutaneous melanomas but not in intracranial brain metastases [23]. Similarly the anti-EGFR antibody Cetuximab was ineffective in orthotopic human GBM xenografts when delivered systemically, but potently blocked tumor growth in the brain when applied via an osmotic minipump [24]. There is also convincing

evidence that small molecule inhibitors like erlotinib do not efficiently reach the invasive tumor cells in the brain because of ABC efflux transporters expressed on endothelial cells [25]. Thus although blood vessels are leaky in the tumor core of high grade gliomas, as indicated by the leakage of MRI contrast agents, the main part of the tumor representing the diffusely infiltrating, so-called 'non-enhancing lesion' is largely protected by a functional BBB. Furthermore the high interstitial pressure in the tumor counteracts the diffusion of agents from blood to tumor, further limiting drug distribution. Therefore systemic delivery often requires high drug concentrations which can be associated with severe side effects. Alternative drug delivery routes are therefore actively investigated, and the development of loco-regional treatment options represent attractive opportunities in the treatment of brain tumors.

During the past decade a number of promising systems have been developed to improve the delivery of chemotherapeutics to the brain. These include strategies such as embedding small molecule antitumor agents in liposomes, micelles, or different types of nanoparticles [26]. Local delivery of the therapeutic agents close to the tumor is associated with high bioavailability of the drug and reduced loss of the therapeutic agents, however a remaining challenge in most cases is the limited diffusion of the drug within the brain [27,28]. Here we review several current encapsulation options for chemical and biological therapeutics for either systemic or local delivery and discuss them in view of their potential clinical application in the fight against brain tumors. In the growing realm of biological therapeutics, which often requires production from living cells, an emphasis is given on the potential of cell encapsulation using biodegradable polymers.

4. Nanosize carriers for drug delivery

Nanocarriers like nanosize liposomes, micelles and polymeric nanoparticles are promising systemic drug delivery vehicles (Fig. 1). Although liposomes and micelles can reach micrometer sizes, for therapeutic drug encapsulation they are mostly used in the nanometer range [29]. By encapsulating drugs inside a nanocarrier, the solubility and stability of the drugs can be improved, providing an opportunity to reevaluate potential drugs previously abandoned because of poor pharmacokinetics [30]. The small size allows nanocarriers to overcome biological barriers and achieve cellular uptake. Moreover passive targeting (enhanced permeability and retention (EPR) effect) and active targeting (ligands, which bind to specific receptors on tumor cells) strategies of the nanocarrier can increase the efficacy of delivery of drugs to tumor site and reduce toxic side effects [31]. In addition to systemic delivery, nanocarriers can also be administered locally at the brain by injections or infusions with implantable minipumps (MiniMed®) by convection enhanced delivery (CED), trans-nasal drug delivery or polymeric implants (Wafers-Gliadel®) [28]. Each of these local delivery routes has its own pros and cons which are discussed in detail elsewhere [28].

4.1. Liposomes

Liposomes are artificially prepared vesicles made of lipid bilayers (Fig. 1A). By definition liposomes are spherical, self-closed structures, formed by one or several concentric lipid bilayers surrounding an aqueous phase [32]. The liposome bilayer is mainly composed of natural and synthetic phospholipids and cholesterol. By changing the phospholipid and cholesterol ratios in liposomes it is possible to regulate the release kinetics [33]. Liposomes can vary in size from the low nanometer range to tens of micrometers, and have several attractive properties for pharmacological applications. For example liposomes can entrap water-soluble hydrophilic agents in their internal water compartment and water-insoluble hydrophobic drugs in the membrane (Fig. 1A). This provides a unique opportunity to deliver different types of pharmaceuticals into cells or even inside individual cellular compartments.

For optimal loading and delivery, the drug must be compatible with the liposome structure and should allow efficient loading into the liposomes. Pharmacokinetics and bioavailability of liposome-based drugs depend on size, charge, membrane lipid packing, and steric stabilization, as well as on the administered dose and route of administration [33]. A major limitation in the efficacy of liposomes is their fast elimination by the cells of the mononuclear phagocyte system (also known as reticuloendothelial system). These cells, primarily monocytes and macrophages, have phagocytic capacities that can take up and degrade liposomes before they reach the tumor. To avoid this type of degradation liposomes are often coated with stabilized hydrophilic polymers such

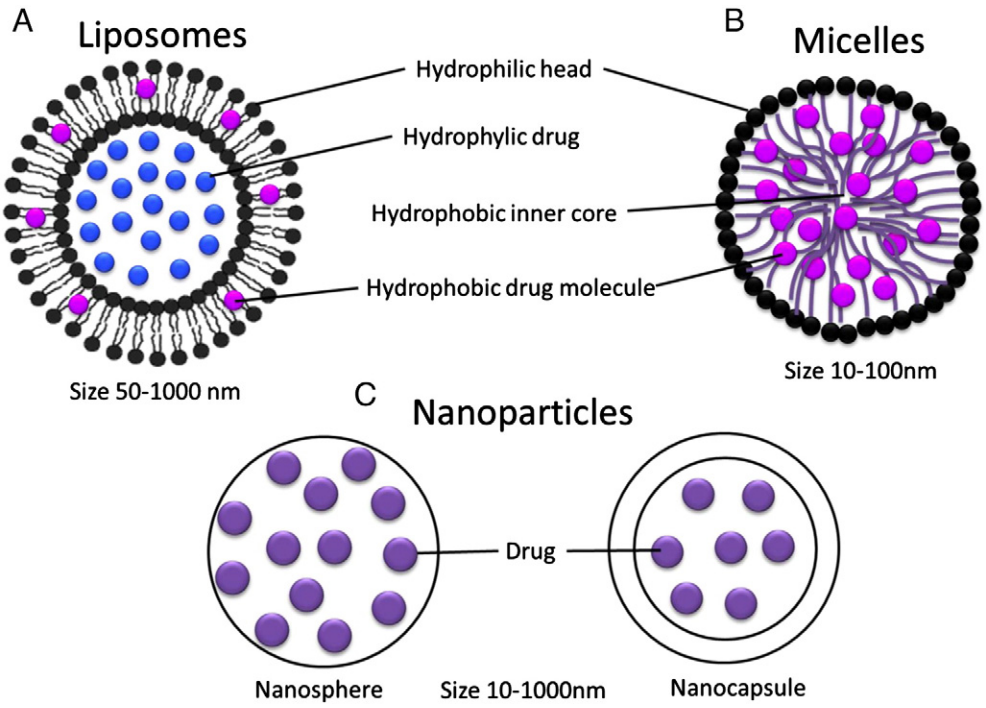


Fig. 1. Schematic illustration of (A) liposomes, (B) micelles, (C) nanoparticles: nanospheres left, nanoparticles right.

as polyethylene glycol (PEG). PEG coating ('PEGylation') causes sterical hindrance for uptake by mononuclear phagocytes and also avoids interaction with blood plasma components, reduces renal filtration, and improves solubility, thus improving the pharmacokinetic, pharmacodynamic, and the immunological profile of drugs in the liposomes [34]. Although PEGylation prolongs the circulation time, the PEG moiety does not actively target the liposome to the tumor. In order to further improve the specificity of liposomes and bioavailability of the enclosed drugs, PEGylated liposomal surfaces can be coupled with antibodies to facilitate tumor specific delivery of the liposomes [35]. Mamot et al. showed that liposomes coated with antibodies (immunoliposome) against EGFR enhanced the targeting of multiple anticancer drugs in tumor models in vivo [36]. The same group recently reported a phase 1 clinical trial (NCT01702129) to address the safety and pharmacokinetics of EGFR antibody coated liposomes loaded with doxorubicin against solid tumors [37]. Efforts are also being made to improve the passage of liposomes through the BBB by e. g. coating the tips of PEG with glutathione, an endogenous anti-oxidant that is actively taken up by specialized transporters in brain endothelial cells [38]. A phase 1/2 clinical trial is currently ongoing to determine the effect of glutathione PEGylated liposomal doxorubicin (2B3-101) in brain tumor patients (NCT01386580, see also www.tobbb.com).

Table 1 summarizes the recent clinical trials using liposomal drug delivery systems to treat different types of brain tumors as registered on www.clinicaltrials.gov [39]. These trials show the principle applicability of liposome-based therapies for brain tumors. For example a randomized clinical phase 4 study (NCT00029523) in patients with neoplastic meningitis investigated the effect of liposomes carrying cytarabine (DepoCyt) versus traditional methotrexate therapy [40]. While the extent of adverse side effects was comparable between the two groups, DepoCyt produced a response rate comparable to that of methotrexate but significantly increased the time to neurological progression (58 versus 30 days). A similar phase 1/2 study (NCT00944801) was carried out with pegylated liposomal doxorubicin combined with prolonged administration of temozolomide and radiotherapy. This study confirmed the safety and feasibility of the approach, without however indicating improved treatment effects [41]. Several of these studies are still ongoing and results are not available yet. Up to now the overall conclusion is that administration of liposome encapsulated drugs is tolerable and feasible for the treatment of brain tumors.

Nevertheless a number of biological and technical challenges remain to be overcome before the technology can be considered for widespread clinical application. Technical issues that need to be solved include a limited loading capacity, the low carrier stability, and the high costs of preparation [42]. In addition the delivery through the BBB needs to be improved. Approaches to do so include chemical modifications of the liposomal surface by incorporation of positively charged lipids, by conjugating surfaces with stimuli-sensitive polymers, by the attachment of cell-penetrating peptides, or the incorporation of viral components [43]. Another major challenge is to find adequate strategies to directly target tumor cells such as tumor specific antibodies. Like most solid tumors, brain tumors do not express unique antigens but share them with normal tissue. A potential candidate for tumor targeting is EGFR and its most common variant EGFRvIII which is characterized by deletion of 267 amino acids in the extracellular ligand binding domain. EGFR is overexpressed in more than 40% of GBMs the majority of those also express EGFRvIII which is specific for tumor cells, making it an ideal candidate for targeted therapy [44]. Monoclonal

Table 1
List of clinical brain tumor trials with liposomal carriers registered on www.clinicaltrials.gov.
Abbreviations used: P — phase, DR — delivery route,, IV — intravenous, IT — intrathecal, O — orally, NPE — number of patients enrolled, AG — age groups, A — adult, C — child, S — senior.

Nos	Liposome drug	Purpose	P	DR	NPE	AG	NCT number	Status
1	Cytarabine	To demonstrate the safety of whole brain radio therapy (WBRT) administered at the same time as intrathecal liposomal cytarabine (depocyte®) versus intrathecal liposomal cytarabine (depocyte®) administered after WBRT for the treatment of solid tumor neoplastic meningitis in patients with or without brain metastasis.	1	IT	18	A/S	NCT00854867	Completed, publication awaited
2	Cytarabine	To test the safety and tolerance of the combination therapy with cytarabine, lomustine and whole brain radiotherapy in patients with leptomeningeal metastasis from malignant melanoma.	1	Injected near spinal cord	9	A/S	NCT01563614	Active, not recruiting
3	Cytarabine	Aims to develop a new treatment for GBM by suppressing glial progenitor cells that surround the ventricular system in patients with these aggressive tumors because it is these regions that appear to act as an incubator for future recurrences resulting in patient death.	1/2	IT	29	A/S	NCT01044966	Recruiting
4	Cytarabine	To study how well giving high-dose methotrexate together with liposomal Cytarabine works in treating patients with central nervous system (CNS) metastases from metastatic breast cancer.	2	IV	22	A/S	NCT00992602	Recruiting
5	Cytarabine	To find out how well an experimental drug called DepoCyt works for neoplastic meningitis	4	IT	100	A/S	NCT00029523	Completed [40]
6	Doxorubicin hydrochloride	To study the effectiveness of liposomal doxorubicin in treating children who have refractory solid tumors.	1	IV		C/A	NCT00019630	Completed, publication awaited
7	Glutathione PEGylated doxorubicin	The purpose of this study is to determine the safety, tolerability, and pharmacokinetics (PK) of 2B3-101 both as single agent and in combination with trastuzumab. Furthermore, the study will explore the preliminary anti-tumor activity of 2B3-101 as single agent in patients with recurrent malignant glioma as well as in patients with various forms of breast cancer with and in combination with trastuzumab in HER2+ breast cancer patients with brain metastases.	1/2	IV	55	A/S	NCT01386580	Recruiting
8	PEGylated doxorubicin	To determine the dose limiting toxicity of PEG-Dox combined with prolonged administration of TMZ.	1/2	O	63	A/S	NCT00944801	Completed [41]
9	Vincristine sulfate	To determine the safety and efficacy of Marqibo as a treatment for children who have been diagnosed with certain types of malignant cancer that has not responded to standard treatment.	1/2	IV	60	C/A	NCT01222780	Recruiting

antibodies like cetuximab, mAB 528, mAB 806, Y10, L8A4 targeting EGFR and/or EGFRvIII are currently used in clinical and pre-clinical studies [44]. Another caveat is that most antigens are not homogeneously expressed throughout the tumor and their expression may change over time. Some antigens may be shed or secreted, leading to potentially high levels of soluble antigen that could make the specific targeting less efficacious [45]. Nevertheless tumor cells may express these molecules at higher levels, which may be sufficient for preferential targeting thus increasing the therapeutic window [45]. The major biological challenge is to identify efficient targets on brain tumors, an issue which basically applies to all of the approaches involving drug carrier systems discussed below.

4.2. Micelles

Micelles are formed when amphiphiles are placed in water. Amphiphiles are compounds possessing both hydrophilic and lipophilic properties. Upon contact, the nonpolar water environment forces the amphiphiles to form micelles by pushing the nonpolar portions into the interior and the polar portions onto the exterior surface [46]. The hydrophobic core of micelles serves as a depot for poorly water-soluble drugs, whereas the outer hydrophilic shell can protect encapsulated drugs and prolong their blood circulation time [46] (Fig. 1B). The use of synthetic and biodegradable polymer-based micelles as drug carrier has gained much attention by the scientific community because of their solubilization, selective targeting, high degree of compatibility with the host, their favorable degradability, and the multiplicity of functional groups they display for the conjugation of drug molecules, P-glycoprotein inhibition and altered drug internalization routes and subcellular localization properties [47,48]. Polymeric micelles are based on block copolymers (comprising two or more homopolymer subunits linked by covalent bonds) with hydrophilic and hydrophobic units that self-assemble in an aqueous environment into micellar structures [49]. Polymer selection for micelles is based on the characteristics of both the hydrophilic and the hydrophobic block copolymer [49]. PEG is the most commonly used hydrophilic polymer. The hydrophobic component of a block copolymer should possess a high drug loading capacity and optimal compatibility of the hydrophobic core with the incorporated drug. Most commonly used polymers for hydrophobic core formation component are polyesters, polyethers, and polyamino acids [49]. Frequently used hydrophobic core-forming molecules are poly(propylene oxide) (PPO), poly(D,L-lactic acid) (PDLLA), poly(ε-caprolactone) (PCL), poly(L-aspartate) and poloxamers [49]. The hydrophobic component of micelles provide a natural carrier environment that allows for encapsulation of poorly soluble anticancer drugs such as lomustine, carmustine, temozolomide and 5-fluorouracil used against brain tumors [50]. Micelle can target tumors areas by two main pathways either via the enhanced permeability and retention (EPR) effect where micelles diffuse passively through disrupted BBB to reach glioma cells or via interaction with endothelial cells and transcytosis to the tumor parenchyma [51]. Drug release from micelles at the targeted area can be enhanced by applying an internal or external trigger. Several methods for triggered release have been described, including pH- and thermo-sensitivity, ligand-mediated monoclonal antibodies (mAbs) or their Fab fragments, oligosaccharides or peptides coupled to the hydrophilic end of the micelle [47,52]. Micelles which are conjugated with specific ligands like folic acid, transferrin, cRGD (cyclic Arg-Gly-Asp) peptide, NGR (Asn-Gly-Arg) peptide, α 2 glycoprotein can bind to the corresponding receptors that are over expressed on the tumor cell surface [51,53]. As a result micelles can be internalized by receptor mediated endocytosis and thus increasing intracellular drug concentration.

Some micelle based drug delivery systems have entered the stage of clinical testing. Although no such studies have been performed in brain tumors yet, the results obtained with other cancers are encouraging and to our opinion merit consideration for application in the treatment of brain tumors. Table 2 summarizes recent clinical trials [39] with micellar drugs in various cancers including breast, pancreatic, lung, urethral, bladder and ovarian cancer. Most studies address the efficacy of paclitaxel-encapsulated micelles (Genexol-PM) compared to regular paclitaxel (Genexol) and demonstrate the safety and feasibility of the micellar drug. In some cases a more efficacious response was observed compared to traditional therapies [54,55], while other studies are not conclusive yet.

The main disadvantages with most micelle carriers are the limited stability, difficult polymer synthesis and immature drug incorporation technology, limitation to hydrophobic drugs, slow extravasation, and possible chronic liver toxicity due to slow metabolic processing [56].

4.3. Polymeric nanoparticles

Polymeric nanoparticles are very small carrier systems ranging from 10 to 1000 nm in diameter. They are made of different types of natural or synthetic polymers, which are generally biodegradable. Examples of natural polymers used for making nanoparticles include cellulose, gelatin, pullan, alginate, gliadin, and chitosan [57]. Synthetic biodegradable polymers are polylactide (PLA), poly-(lactide-co-glycolide) (PLGA), polyanhydrides, poly-ε-caprolactone, and polyphosphazene [57].

Table 2
List of clinical trials with micellar carriers for various cancer, registered on www.clinicaltrials.gov.
Abbreviations used: P — phase, DR — delivery route, IV — intravenous, NA — not available, NPE — number of patients enrolled.

Nos	Micelle drug	Cancer type	Purpose	P	DR	NPE	NCT number	Status
1	Drug: paclitaxel loaded polymeric micelle (Genexol-PM).	Breast cancer-recurrent	To evaluate the response rate in patients with taxane-pretreated recurrent breast cancer receiving paclitaxel loaded polymeric micelle (Genexol-PM).	4	IV	90	NCT00912639	Enrolling by invitation
2	Drug: NK105; drug: paclitaxel	Breast cancer — metastatic or recurrent	To verify the non-inferiority of NK105, a paclitaxel-incorporating micellar nanoparticle, to paclitaxel in terms of the progression free survival in patients with metastatic or recurrent breast cancer.	3	IV	380	NCT01644890	Recruiting
3	Drug: Genexol-PM	Bladder cancer, ureter cancer	To explore the efficacy and safety of Genexol-PM in advanced urothelial patients, who previously treated with gemcitabine plus platinum as adjuvant chemotherapy or 1st line therapy for metastatic diseases.	2	IV	37	NCT01426126	Completed [54]
4	Drug: Genexol-PM/ gemcitabine	Non-small cell lung cancer	A phase 2 trial of Genexol-PM and gemcitabine in patients with advanced non-small-cell lung cancer	2	NA	45	NCT01770795	Completed, publication awaited
5	Drug: paclitaxel loaded polymeric micelle	Pancreatic cancer	To study how well paclitaxel works in treating patients with unresectable locally advanced or metastatic pancreatic cancer.	2	IV	43	NCT00111904	Completed [55]

Synthetic polymers are preferred by some because of the predictable chemical and physical properties, such as solubility, permeability, and rate of degradation. Drugs can be adsorbed, dissolved, entrapped, encapsulated or covalently linked to the nanoparticles [58]. Nanoparticles can be generally classified into two different types: nanospheres and nanocapsules (Fig. 1C). In nanospheres, drugs are either adsorbed or entrapped within the polymeric matrix, whereas in nanocapsules, drugs are confined to the inner liquid core while the polymeric membrane covers the external surface of the particle [58]. Because of the various capsule design properties, polymeric nanoparticles become more popular in drug delivery. Drugs that have successfully been transported into the brain of animals by means of nanoparticles include the hexapeptidedalargin, the dipeptide kytorphinloperamide, tubocurarine, the N-methyl-D-aspartate (NMDA) receptor antagonist MRZ 2/576, and doxorubicin [59]. Recently magnetic nanoparticles (MNPs) have been proposed for application in brain tumor imaging and therapy [60].

Although systemically administered nanoparticles are chemically more stable than liposomes and micelles, nevertheless many nanoparticles are lost due to phagocytic activity of the mononuclear phagocyte system [58]. The other drawbacks of nanoparticles for drug delivery are the complexity of the preparation and encapsulation procedures, high manufacturing cost, and the risk that immune response and allergic reactions may be triggered [61,62]. The future of nanomedicine will depend on the rational design of biocompatible polymeric material and a better understanding of biological processes and associated risks.

5. Cell carriers for biologics

5.1. Concept of cell encapsulation

Encapsulation of cells producing therapeutic molecules (peptides or proteins) offers a local and continuous drug delivery system and is a promising strategy for therapeutic applications in the brain [63–65]. Cell encapsulation involves the entrapment of living cells such as fibroblasts, myoblasts, embryonic or mesenchymal or neural stem cells [66] engineered to produce a therapeutic molecule within a semipermeable membrane. This semipermeable membrane allows bidirectional diffusion of molecules, such as influx of nutrients, oxygen, and outflux of therapeutic molecules and metabolic waste products, while at the same time the membrane protects the foreign cells from the host immune system (Fig. 2A). Cell encapsulation also holds the advantage of delivery of the therapeutic agents for prolonged periods of time without the necessity to repeat the treatment. This approach may be particularly applicable for aggressive gliomas. It is estimated that 80–90% of these tumors recur within 2 cm of the original resection site [67]. By implanting encapsulated recombinant cells producing the therapeutic agent in the immediate vicinity of tumor, recurrence of gliomas may be delayed. Cell encapsulation is likely to raise increased interest in the current era of biological therapeutics that cannot be produced through chemical processes, but require living organisms to obtain an active compound.

5.2. Polymers applied for cell encapsulation

An important consideration is which materials qualify for implantation in the brain. The material should not be associated with neurotoxicity and should be compatible with the survival of the encapsulated cells, both during the encapsulation process and during long term maintenance of the capsules. Similarly, the capsules should not evoke an inflammatory response at the implantation site that could harm either the encapsulated cells and/or the host organ. To avoid interference with the survival of the therapeutic cells, harsh chemicals or high temperatures have to be avoided during synthesis or crosslinking [68]. As a consequence many encapsulation procedures disqualify since only those that allow for encapsulation under mild, physiological conditions are suitable for cell encapsulation. The majority of such procedures are those that form hydrogels. These procedures usually require polymers

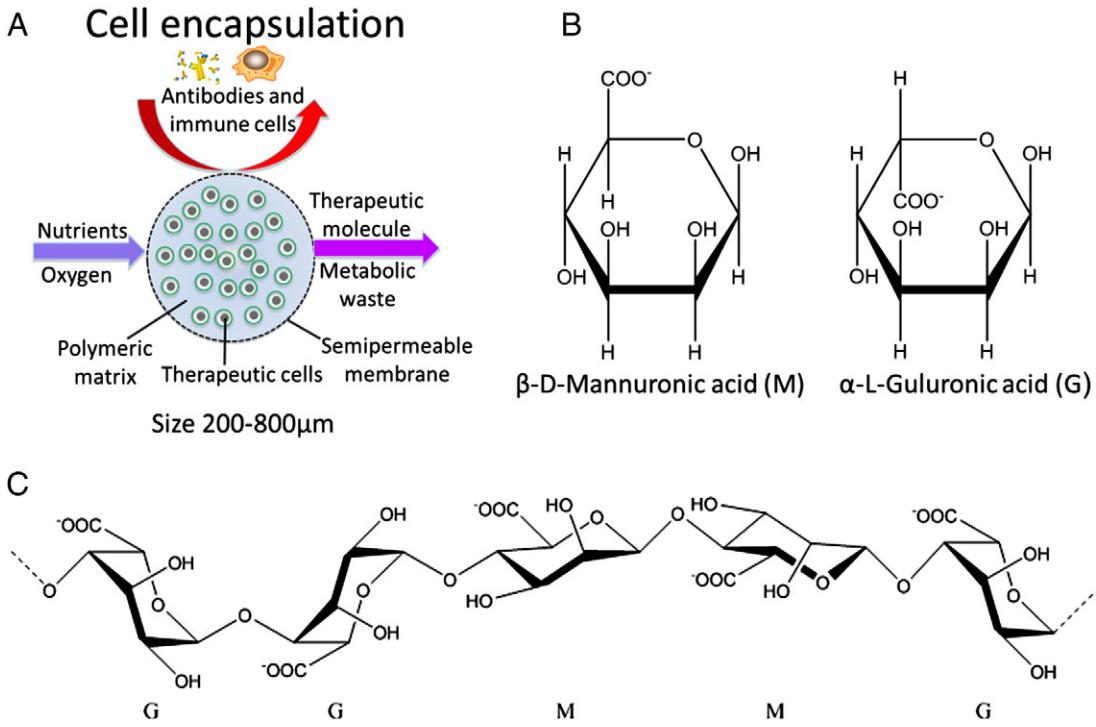


Fig. 2. Schematic illustration of (A) encapsulation of living cells. In cell encapsulation technology, nutrients, oxygen, metabolic waste, and therapeutic molecules diffuse across the polymeric semipermeable membrane. The semipermeable membrane also protects encapsulated cells from the host immune system. (B)The structure of monomeric subunits in alginate. Alginate molecules are linear block copolymers of beta-D-mannuronic (M) and alpha-L-guluronic acids (G). (C) These monomers are linked by 1–4 linkage in a pattern of blocks like GG block, MM block and MG block.

from natural sources but also some synthetic sources have been proposed. The most commonly applied polymers are alginate, hyaluronic acid, chitosan, agarose, cellulose sulfate, polyethylene glycol, polymethacrylate [69,70]. All the polymers applied for encapsulation are discussed in detail in another paper in this issue [71].

Although the choice of polymer best suited for clinical application in the brain has not been settled yet, alginate is so far the most intensively studied polymer for biomedical applications. The reason is that alginate is well-characterized, non toxic, and also highly compatible with the microenvironment in the brain [72,73]. Also alginate capsules have been reported to survive for many months in animal models in the absence of adverse effects in the host [74–77]. This is a major advantage as it allows long term treatment and eliminates the need to remove the capsules from the implantation site after the graft ceased to function. Chemically, alginate is an unbranched binary copolymer of (1–4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Fig. 2B–C). Based on the composition of monomers, alginates are classified as high G alginate, intermediate G alginate and low G alginate.

An important parameter to consider is the inflammatory response of the brain to implanted biomaterials [78]. Incomplete knowledge is available about such responses [79]. Previous studies form our group showed that astrocytes play an important role in responses and may surround/envelop capsules leading to insufficient supply of nutrients and induction of necrosis in the encapsulated cells [80]. These responses can be delayed and even prevented by applying highly purified alginates [81] and capsules with a sufficient rigidity [73,82]. Rigidity requires some special consideration, as capsules in the brain are exposed during and after implantation to high shear forces. To withstand these forces it is preferable to apply alginates that form solid, rigid gels [83]. The strength of the alginate is strongly correlated with the polymer composition. G residues have higher affinity for crosslinking cations than M residues, therefore high G alginate forms rigid gels which can better withstand the shear forces in the brain [73].

5.3. Therapeutic modalities amenable to cell encapsulation

Cell encapsulation is applicable to delivery of cell-secreted products. In the era of targeted treatment options, more and more therapeutics are of biological origin are under investigation. These include proteins, peptides, antibodies, nucleic acids and viral vectors [84]. Here we provide examples of therapeutic modalities of particular interest for brain tumors that could be delivered by cell encapsulation technology. Some of them have been tested in clinical trials or in pre-clinical animal models (Table 3).

5.3.1. Proteins

5.3.1.1. Antibodies. Blocking antibodies or antibody fragments against growth factor receptors and/or their ligands. Examples of antibody-based drugs are Cetuximab, an anti-EGFR antibody and Bevacizumab, an antibody against vascular endothelial growth factor (VEGF). Both drugs are already in clinical use for glioma treatment [85–87]. Their efficacy might be substantially enhanced by local delivery.

5.3.1.2. Soluble receptors. Soluble receptors interfering with growth factor receptor signaling: VEGF Trap (Aflibercept) composed of the extracellular parts of VEGF-R1 and VEGF-R2, is an anti-vascular agent acting as a trap for its ligand VEGF [88]. The soluble form of the stem cell regulator LRIG1 inhibits glioma growth by interfering with receptor tyrosine kinase signaling [65].

Table 3
Pre-clinical and clinical studies on cancer using cell encapsulation technology.

Cancer type	Cell line and agent	Modality	Comment
Glioblastoma	Intracerebral implantation of encapsulated human fetal kidney 293-EBNA (Epstein–Barr virus nuclear antigen) cells overexpressing in rats	Endostatin —endogenous inhibitor of angiogenesis	Prolong survival [63]
	Baby hamster kidney (BHK) overexpressing human endostatin (hES) implanted in mouse xenograft model	Endostatin —endogenous inhibitor of angiogenesis	Reduced tumor growth [92]
	Subcutaneous and intracerebral implantation of encapsulated human fetal kidney 293 cells (293-EBNA) over expressing in mice	Endostatin —endogenous inhibitor of angiogenesis	Reduced tumor growth and invasion [93]
	Intracranial implantation of mouse neural stem cells expressing s-TRAIL (secretable tumor necrosis factor apoptosis inducing ligand in xenograft GBM mice model	Peptide-s-TRAIL — targeted apoptosis-induction	Reduced tumor growth and prolong survival [89]
	Intraventricular implantation of BHK cells overexpressing Lrig1 (leucine-rich repeats and immunoglobulin-like domains 1) in glioblastomaxenograft mice model	Peptide-sLRIG1 — blocking antibody (EGFR)	Reduced tumor growth and prolong survival [65].
	Psi2-VIK cells encapsulated in microporouspolyethersulphone(PES) and implanted in stratium of C6 glioblastoma bearing rats	Retrovirus — herpes simplex virus-1 thymidine kinase (HSV-TK)	Increases tumor necrosis [95]
Leukemia	Anti-p15E antibody-producing hybridoma cells were encapsulated in alginate and injected intraperitoneally in tumor-bearing mice.	Protein-anti-p15E — immuno-stimulatory proteins	Showed inhibition of tumor cell growth, significant longer survival [111].
Colon cancer	Inducible nitric oxide synthase (iNOS) expressing cells, in a xenograft nude mouse model	Protein-iNOS — immuno-stimulatory protein	Prolong survival [112]
	Mouse fibroblasts NIH3T3 expressing murine interleukin-12.	Protein-interleukin-12 — immuno-stimulatory protein	Induce potent anti-tumor immune response and constitute an efficacious therapy [113]
Ovarian cancer	Inducible nitric oxide synthase (iNOS) expressing cells, in a xenograft nude mouse model.	iNOS — immuno-stimulatory protein	Curative treatment [112].
Pancreatic cancer	Genetically modified allogeneic cells, which expressed a cytochrome P450 enzyme.	Immune-modulatory peptide — Cytochrome P450	Tumor suppression: Clinical trials in patients with inoperable pancreatic carcinoma [114]

5.3.2. Peptides

5.3.2.1. Anti-angiogenic peptides. These include endogenous inhibitors of angiogenesis known to be present in the brain (angiostatin, endostatin, PEX, pigment epithelial-derived factor, and thrombospondin (TSP)-1 and 2). An inhibitory peptide against integrins (Cilengitide) has also anti-angiogenic activity and is in clinical studies for GBM.

5.3.2.2. Apoptosis inducing peptides. The peptide TRAIL (TNF-α related apoptosis inducing ligand) induces ligand-mediated apoptosis and has shown promising results in animal models [89].

5.3.2.3. Endogenous differentiation agents. Examples are neurotrophic factors (e. g. nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF) or neurotrophin-3 (NT3)). Such agents may potentially block mitogenic signals in cancer cells through induction of a cellular differentiation process.

5.3.2.4. Immune-modulatory peptides. Release of immune-stimulatory cytokines to circumvent the immuno-suppressive effect of the tumor.

5.3.3. Viral vectors

Viruses produced from encapsulated packaging cell lines can deliver suicide genes to the tumor cells such as pro-drug converting enzymes (e. g. HSV-thymidine kinase gene). Oncolytic viruses can be released from producer cells.

5.3.4. Nucleic acids

5.3.4.1. RNA. Several novel drug modalities are based on small non coding RNAs (short hairpin RNAs, micro RNAs) which may affect specific gene transcription or regulate whole signaling networks. In the future it may become possible to design RNA molecules including long non coding RNAs (lncRNAs) to be released from the cell which may offer hitherto unforeseen opportunities for therapeutic application.

All of these biological drugs can be applied alone or in combination with systemically administered drugs, cell-based immunotherapy or other treatment modalities. Microencapsulation also provides the possibility of encapsulating different producer cell lines within one capsule or cells engineered to overproduce different biological drugs at the same time.

5.4. Proof-of-concept of cell encapsulation therapy for malignant brain tumors

The concept of cell encapsulation for the treatment of brain tumors has already been proposed many years ago [27,90,91]. The approach seems straightforward: at the time of surgery after debulking the tumor mass, the encapsulated cells producing the therapeutic agents could be implanted in the immediate vicinity of the excised tumor (Fig. 3). These implanted encapsulated cells receive nutrients and oxygen supply from the interstitial fluid and are protected from host immunity by the semipermeable membrane. These implanted encapsulated cells can than function for prolong period of time producing anti-tumor agent, inhibiting malignant cells that cannot be excised by surgery. This strategy holds a number of important advances over conventional chemotherapy and encapsulation of chemicals. The release of the therapeutic agent is limited to the tumor area without undesired side effects in surrounding healthy brain and other organs.

Several pre-clinical studies have shown the feasibility and efficacy of cell encapsulation technology in brain tumor models. In 2000 Read et al. showed that the anti-angiogenic peptide endostatin secreted from alginate capsules led to a considerable survival benefit in the immunecompetent BT4C rat brain tumor model [63]. Endostatin was readily detected in the cerebrospinal fluid indicating that therapeutic substances can distribute throughout the brain from the intraparenchymal transplants. Importantly no major immune reaction against the alginate beads was observed and no antibodies against human endostatin were detected in the serum. Similar results were published in a subcutaneous mouse model using endostatin expressing BHK cells encapsulated in alginate [92]. Local delivery of endostatin produced from alginate encapsulated 293 EBNA cells was also found to reduce glioma induced angiogenesis with regard to vascular density, morphology, and functionality [93]. Unfortunately clinical trials using systemic delivery of endostatin have been disappointing [94], which may be partially due to the short half life of the peptide and its poor distribution. The

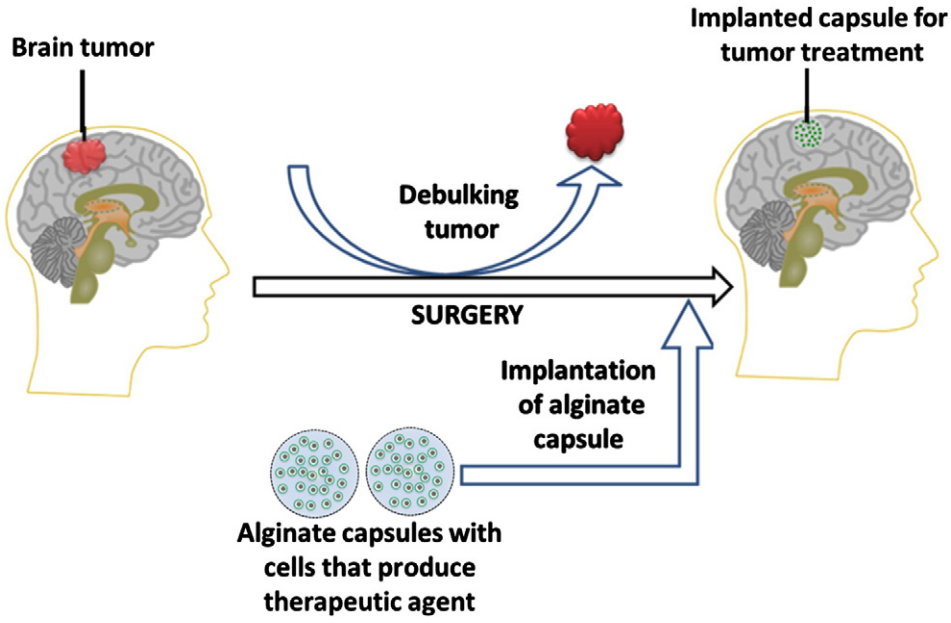


Fig. 3. Proposed treatment for brain tumors by cell encapsulation technology. At the time of surgery, when the tumor is excised, the surgeon can implant in the immediate vicinity of the excised tumor one or more capsules containing cells that produce the therapeutic agents. Since the release of the therapeutic agent is local undesired side effects from systemic administration are prevented. Importantly, the drugs are produced over prolonged periods of time and can target the remnant of malignant cells not eliminated by surgery.

pre-clinical data suggests that the release from encapsulated cells may be more effective than other methods of administration.

Targeted induction of apoptosis from encapsulated cell factories is an elegant approach to target brain tumors [73]. A recent promising study in mice has shown that intracranial implantation of therapeutic stem cells overexpressing secretable TRAIL, encapsulated in biodegradable synthetic extracellular matrix, delayed tumor regrowth and significantly increased survival of mice bearing established GBMs [89]. Synthetic polymers (polyethersulphone membrane) have also been used to encapsulate retrovirus packaging cell lines implanted in a syngeneic rat GBM model [95]. Retroviral-mediated gene transfer of the suicide gene thymidine kinase led to a substantial increase in tumor necrosis although only a limited number of glioma cells were transduced (3–5%). Nevertheless transduction efficacy was increased compared to direct injection of viral particles, which can probably be explained by the continuous delivery of the vectors.

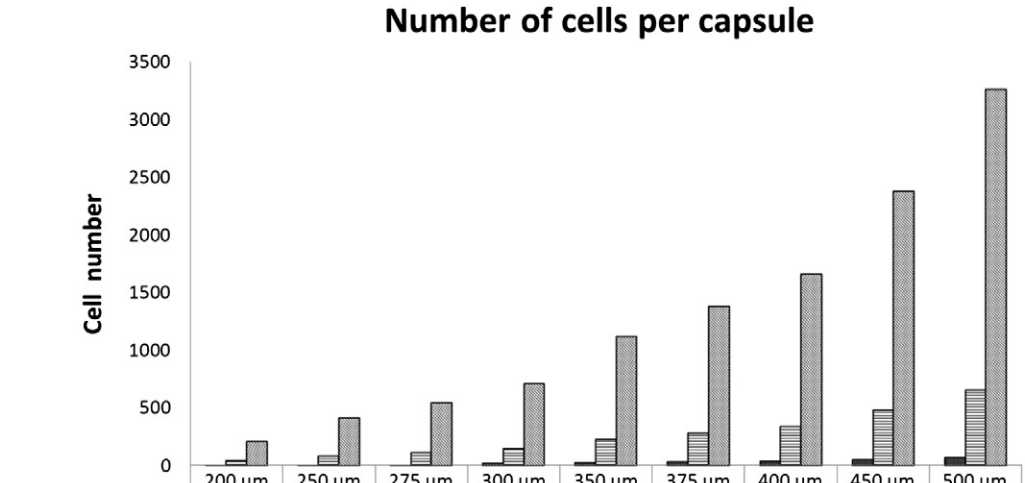
Our group recently demonstrated the power of alginate cell encapsulation therapy in human GBM derived xenograft models in mice. Cells were engineered to overexpress the extracellular part of LRIG1 (leucine-rich repeats and immunoglobulin-like domains 1 protein) a tumor suppressor protein known to regulate EGFR signaling [96]. Implantation of alginate capsules into the mouse brain led to potent inhibition of glioma growth and increased survival of glioma-bearing mice, even when the implants were engrafted after tumor establishment [65]. Similar experiments have also been successfully performed for other brain disorders [77,97] and importantly proof-of-concept studies in minipigs have been reported by two independent groups [64,98]. HEK cells encapsulated in alginate could be successfully recovered with minor host response up to 5 months after implantation in minipig brains. Beads, which were located close to the meninges at the brain surface, were overgrown by fibroblast-like cells, but not those in the brain parenchyma [64]. Fjord-Larsen et al. succeeded in encapsulating nerve growth factor (NGF) secreting cell line in polyether sulphone-based macrocapsules (clinical device named NsG0202) and retrieving NsG0202 after 12 months from basal forebrain of mini-pigs. The study showed the device was implanted and retrieved without complications and was well tolerated, also the device contained a high number of viable cells [71]. This has led to a recent phase 1 clinical trial in Alzheimer patients using NGF secreting cells [99].

5.5. Challenges of cell encapsulation

A major challenge in cell encapsulation is obviously the selection and availability of the appropriate therapeutic agents. In addition to be highly effective against the tumor cells, the therapeutic agent should not be too large in order not to exceed the molecular cut-off of the applied semipermeable membrane. Although the cut-off size of the membrane can be adapted, it should provide protection against immunoglobulins and thus the therapeutic agent should not exceed the molecular weight of the smallest immunoglobulin's (150 kDa) [100]. Efforts should be made to identify and apply the smallest moiety of the protein with active therapeutic activity. The smaller the molecule the better will also be the diffusion in the brain parenchyma.

Other challenges include capsule size, stability and long term survival of the encapsulated cells. Many of these parameters are linked, e. g. the capsule stability increases with capsule size but decreases with cell load (unpublished data). Fig. 4 shows the cell loading density of capsules in relation to the diameter of the capsules. Using 50 million cells per ml in the starting solution, will deliver 205 cells in 200 μm capsules, and 3260 cells in 500 μm capsules. Capsules larger than 800 μm in diameter are associated with reduced diffusion of nutrients and oxygen inside the capsule core, which leads to starvation and necrosis of encapsulated cells [101]. Small capsules (200–400 μm) are ideal for rodent models, but may not be optimal for the human brain where large-sized retrievable macro capsules may be preferred for safety reasons. Alternatives may be the production of elongated capsules with a small diameter, thus still providing optimal perfusion. Most clinical trials implanting capsules into the human brain have used elongated capsules produced from synthetic polymers [97,99]. In any case the determination of the optimal capsule size and cell load is a prerequisite for long term application.

Although high cell numbers may be encapsulated without interfering with the mechanical stability of the capsules, this is often associated with protrusion of cells. This however represents a major safety concern for clinical application. Cancer patients are often immunocompromised [102] and may have reduced responses against cells leaking out of the capsules, which harbors the risk of development of neoplasms. Preventing cell leakage is even more important in the brain, which is an immune privileged site. Protrusion of cells from alginate capsules is



	200 μm	250 μm	275 μm	300 μm	350 μm	375 μm	400 μm	450 μm	500 μm
1 million cells/ml	4	8	11	14	22	28	33	48	65
10 million cells/ml	41	81	108	141	223	275	331	475	652
50 million cells/ml	205	405	540	705	1115	1375	1655	2375	3260

Fig. 4. Relationship showing the number of cells per capsule as a function of capsule size. As the capsule size increases there is an exponential increase in cell number encapsulated per capsule. For example starting with a solution of 10 million cells per ml of polymer matrix, a capsule of 200 μm carries 41 cells, while a capsule of 400 μm carries331 cells (which is eight times more than the capsule size of 200 μm).

difficult to prevent with the current encapsulation approaches [103]. In order to reduce outgrowth of cells from capsules and to increase mechanical stability, alginate capsules are often coated with polycations such as poly-L-Lysine [104], poly-L-ornithine [105], poly-methylene-co-guanidine [106] or poly-ethyleneimine [107]. Also recently photosensitive coating with poly-allylamine alpha-cyanocinnamylideneacetate [108], short-chain alginate-co-MPEG (methoxy polyethylene glycol) [109] and N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) [110] have been used to increase the capsule stability and prevent escape of encapsulated cells. Optimal safety and quality controls will be mandatory in order to fulfill the regulatory issues related to the implantation of foreign genetically modified cells into the human body.

6. Concluding remarks

Although several potentially efficacious drugs are available for treating brain tumors, most of them lack efficacy because of poor delivery through the BBB. Liposomes, micelles, and polymeric nanoparticles provide novel ways to deliver drugs either systemically or locally through direct infusion. A limitation of most of these encapsulation approaches is their low efficacy in encapsulation, the systemic toxicity of the drugs, and the high cost of the encapsulation production process. For the in situ delivery of biological molecules, cell encapsulation provides a promising alternative, with the advantage of BBB circumvention, long term release of the active therapeutic molecule and reduced side effects. Also, cell encapsulation allows for tailor made therapies where patient specific and combinatorial components can be applied depending on the genetic makeup of the malignancy. Several encapsulation matrices may be considered, and recent data from pre-clinical and clinical work indicate that this approach merits consideration for future brain tumor treatment.

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