Ligand-mediated transport of drug delivery devices across the blood-brain barrier
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Chapter 8

Summary and Perspectives
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The blood-brain barrier precludes the entry of many drugs into the central nervous system, and in particular that of biological macromolecules, including proteins, peptides, and nucleic acids, that represent an emerging class of potential therapeutics. In the present work, we address this limitation and propose a novel drug carrier, G23-polymersomes, with high transcytotic capacity to transport drugs into the brain.

Ligand-coated nanoparticles hold the potential to target specific tissues, thereby providing a means to improve the bioavailability of drugs. In brain drug delivery, however, the targeted tissue is inaccessible from the systemic circulation, thus requiring a need for nanoparticles to be surface-modified with ligands that display an enhanced affinity for the BBB endothelium.

Currently, ligands that target natural transporters and receptors at the BBB, which are often exploited by pathogens for cell entry, are being exploited to transport drug-containing nanoparticles into the brain (Chapter 2). Antibodies to the transferrin receptor, that bind to epitopes that differ from the binding site of the natural ligand transferrin, can cause accumulation of ligand-drug complexes in the BBB endothelium. However, the transferrin receptor, although abundantly present on the BBB endothelial cells, is not brain-specific. Targeting the insulin receptor, although claimed as a therapeutically active strategy, is likely to cause the receptor’s own sequestration from the luminal face of the BBB endothelium, thereby potentially compromising on brain glucose metabolism. Another class of ligands, Angiopeps, that bind the LDL receptor-related protein (LRP), are already in clinical trials for the treatment of gliomas with i.v. administered Angiopep-paclitaxel complex. Actually, LRP is abluminally sorted in the BBB endothelium, and hence LRP-dependent transport of the complex, as claimed, is unlikely to occur under physiological conditions. Clearly, novel BBB endothelium receptors, that are brain-specific, show an adequate luminal expression, and display the capacity to mediate the transport of nanoparticles, without disturbing transport of their natural ligands, are necessary. In the work presented in this thesis, we focused our search on receptors that, together with a corresponding ligand, are sorted intracellularly for transcytosis, i.e. processed along a transcellular transport pathway from the luminal to the abluminal surface in brain endothelium.

The transcellular transport route across brain endothelium is likely to originate from entry via caveolae, a pathway that is thought to avoid lysosomal delivery. Alternatively, the glycosylphosphatidylinositol anchored protein (GPI-AP) specific uptake route - the GEEC pathway - which similarly avoids lysosomal processing, also constitutes an attractive option for transendothelial transport. By mass spectrometry we identified (Chapter 3) 33 GPI-APs in hCMEC/D3 human brain endothelial cells. The tissue distribution of one of the identified GPI-APs, prion protein (PrP), shows specificity to brain capillaries and the central nervous system (CNS). Moreover, exogenously administered prion is neurotropic and its CNS spreading is dependent on homophilic interactions with endogenous prion. Taken together, these data rationalized our further studies
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Transcellular vesicular transport in BBB endothelium is classified into two types, i.e., receptor-mediated and adsorptive (charge-mediated) transcytosis. These transcytotic transport mechanisms serve to import/export nutrients and metabolites to and from the brain, and may contribute to the brain delivery of nanoparticles. However, the mechanism of transcytosis in endothelial cells is largely unclear. To address this issue, we studied (Chapter 4) the transport of surface-modified nanoparticles in an in vitro model of the BBB – the hCMEC/D3 cell line. 500 nm nanoparticles (NBs) were directed into the caveolar pathway by their (large) size. In addition, prion was covalently attached to the surface of nanoparticles (PrPBs) to induce receptor-mediated endocytosis, while adsorptive transport was provoked by nanoparticles (PEIBs) carrying the positively charged polymer PEI (polyethyleneimine). PrPBs showed the highest transcytotic capacity (6.0 ± 0.9 %) across the in vitro BBB, followed by NBs with 3.4 ± 0.3 %. The uptake of PrPBs and NBs was mainly impeded by caveolae-perturbing agents. However, the intracellular compartments, in which PrPBs and NBs accumulated, are strikingly different. PrPBs reach a sorting compartment, positive for both clathrin and caveolin, as revealed by immuno-fluorescence and electron microscopy, whereas NBs are detected in multivesicular bodies (MVBs) and multilamellar structures. Interestingly, the transcellular transport of low-density lipoproteins (LDLs) - natural particles with a diameter of 22 nm - across the BBB is caveolae-dependent and similarly transverses MVBs. The transcytosis of PEIBs was only 1.3 ± 0.6 %. Although the positive charge strongly promoted uptake of the PEIBs by the endothelial cells, PEIBs remained entrapped in cellular vacuoles and hence, charge-enhanced uptake does not appear to promote transcellular delivery of nanoparticles. Collectively, the results support receptor-mediated routing of nanoparticles via a caveolar pathway for transcellular delivery.

Importantly, the two morphologically distinct compartments, sorting vesicles and multivesicular bodies, represent – at least partly – the pool of the cellular transcytotic compartments.

As we recognized the potential of endogenous PrPc as a receptor for nanoparticles delivery, we next identified novel PrP-binding ligands from a 12-mer peptide phage library (Chapter 5). Peptides are, unlike proteins, easy to produce and require no modifications, such as the humanization of antibodies, at the time of translating animal studies into human therapies. Ligands with affinity for monosialoganglioside GM1, which is enriched in caveolae, were selected as well.

Two prion-binding (Pcs, P9) and two GM1-binding (G88 and G23) peptides were grafted on the surface of polymersomes (polymeric nanoparticles) and transport of these peptide-targeted polymersomes was measured across our in vitro blood-brain barrier cell model. The G23 peptide, selected as GM1 binder, strongly promoted the transcytosis of G23-polymersomes, accounting for 30.8 ± 1.4 % basal recovery of the apically administered dose, which represents an approx.
4-fold increase to the transcytosis of all other peptide-targeted polymersomes tested. Contrary to our expectations, the PrP-binders, Pcs and P9, caused only a limited extent of transcytosis of polymersomes, i.e., of the same order as non-targeted polymersomes (5.7 ± 0.8 %). Interestingly, TAT-peptide failed to produce a significant effect on the transport of polymersomes across the BBB (7.2 ± 1.2 %), although it enhanced the total cellular association of polymersomes. The strong cellular association of TAT-polymersomes may, similar to that of PEIBs, be attributed to its strong positive charge. Yet again, an increase in the uptake of nanoparticles in brain endothelial cells, as induced by a positive charge, not necessarily results in an increase in their transcytosis.

Drug delivery into the brain by the use of ligand-targeted nanoparticles, will require a ligand that not only facilitates the transcytosis of nanoparticles, but also mediates their bioaccumulation at the brain endothelium in vivo. Until now, RI7217-targeted nanoparticles have been described to successfully accumulate in brain vasculature. Therefore, the issue of brain accumulation was addressed by comparing the biodistribution of GM1-targeting G23-polymersomes to that of transferrin receptor-targeting RI7217-polymersomes after tail vein injection in BALB/c mice. The G23-polymersomes accumulated in the brain of mice to an extent similar to that of RI7217-polymersomes, which was significantly higher than the brain accumulation of scrambled G23-polymersomes, signifying the G23 peptide as a ligand with comparable in vivo brain targeting potential as the RI7217 antibody.

Currently, the successful development of nanoparticles for drug delivery into the brain suffers from major data scarcity on the mechanism of transcytosis across the BBB. Thus, clarifying the intracellular processing of G23-polymersomes, that showed affinity for the BBB endothelium in vivo together with high transcytotic capacity in vitro, may provide the mechanistic insight into the process of transcytosis. Therefore, the issue of brain accumulation was addressed by comparing the biodistribution of GM1-targeting G23-polymersomes to that of transferrin receptor-targeting RI7217-polymersomes after tail vein injection in BALB/c mice. The G23-polymersomes accumulated in the brain of mice to an extent similar to that of RI7217-polymersomes, which was significantly higher than the brain accumulation of scrambled G23-polymersomes, signifying the G23 peptide as a ligand with comparable in vivo brain targeting potential as the RI7217 antibody.

To reveal the identity of the cell surface receptor(s) for G23-polymersomes in the hCMEC/D3 brain endothelial cells, competition experiments were carried out using cholera toxin B, a natural ligand for the GM1 ganglioside. Next to GM1, GT1b was identified as a putative receptor for G23-polymersomes. With time G23-polymersomes accumulated in multivesicular compartments, where they colocalized with the transcytosis marker LDL. These data point to MVBs as an intermediate compartment in the transcytotic pathway in endothelial cells, similarly as shown for NBs.
It was speculated that different pathogens and pathogenic derivatives, such as the GM1-binding cholera toxin or the Gb3 (glycolipid)-binding B-subunit of bacterial Shiga toxin, have probably developed a common mechanism based on glycosphingolipid clustering to drive bending of the plasma membrane as an initial step for their endocytosis. Concerning the underlying mechanisms of nanocarrier internalization, G23 might likewise induce lipid reorganization that favors membrane curvature, which drives the formation of vesicles, derived from inwardly curved membranes. Internalization of such re-modeled plasma membranes can generate MVBs for exosome secretion. In light of these findings, it is tempting to speculate an interplay between a receptor and its local lipid environment as a sorting mechanism into transcytotic or degradative pathways. Clearly, the search for receptors to serve in brain targeting should not be limited to transport proteins, as was done so far, but should include (BBB-enriched) glyco(sphingo) lipids, as was shown in this thesis. Moreover, lipids are conserved among species and consequently research in animal models can be directly translated to humans without the need of generating species-specific targeting peptide/antibodies. Attention should be paid to both the brain specificity and the transcytotic potential of the receptors, necessary for accumulation in brain vasculature and transport of nanoparticles across the BBB, respectively.

The natural capacity of neural stem cells (NSCs) to cross the blood-brain barrier has been demonstrated to benefit treatment of multiple neurological disorders (Chapter 7). However, systemic application of stem cells may result in entrapment of cells and unpredicted complications in other organs with high blood supply. To avoid this obvious drawback of cell therapy, but also to make use of the neural stem cells natural affinity for the brain tissue, we finally investigated plasma membrane vesicles (PMVs) prepared from neural stem cells, as alternative drug carriers for the brain. Indeed, NSC-derived PMVs, after systemic administration, showed high brain affinity and significant accumulation in brain cells, indicating their transcytotic capacity. In vitro the PMVs showed a transcytotic capacity comparable to that of the aforementioned G23-polymersomes.

In conclusion, both the targeting of nanoparticles towards natural glycolipids as well as the use of NSC-derived plasma membrane vesicles, representing natural biological nanodevices, open promising new avenues for brain-targeted drug delivery.

**Perspectives**

Demographic surveys in the developed societies show a tendency of prolonged human lifespan, unfortunately paralleled by an increase in the number of neuropathological incidents. The older population is predominantly susceptible to neurodegenerative diseases, like Alzheimer’s and Parkinson’s, whereas the
younger people mainly suffer from anxiety and depression. Symptomatic relief for these conditions, with old generation medications, mostly relies on correction of imbalanced neuromediation and requires prolonged treatment, which is in many cases ineffective. Meanwhile advances in medical research have revealed molecular targets (genes and proteins) for the causal treatment of neurological disorders. Novel therapeutic entities (NTEs), aimed at correction of gene transcription and protein expression, including microRNAs, splice-correcting oligonucleotides, and enzyme-inhibiting antibodies, mostly are macromolecular structures. Hence, these NTEs often show poor cellular penetration due to their size and physiochemical properties, in addition to their susceptibility to enzymatic degradation. Thus, their clinical significance remains low and further improvements are necessary to translate NTEs into ready to use neuropharmaceuticals. Advanced drug delivery systems, in terms of targeted nanoformulations, provide such opportunities.

The classical view of targeted nanoformulations for drug delivery into the brain is represented by \( \leq 200 \) nm nanoparticles, prepared from lipids and/or polymers, and loaded with NTEs. Alternatively, natural sources such as the patient’s own neural stem cells or dendritic cells may be used as “biological” delivery vehicles. The intended accumulation in the central nervous system is achieved via surface-exposed targeting moieties, that enable efficient binding and transport of the nanoparticles across the blood-brain barrier. Interestingly, the G23 peptide has affinity for both GM1 and GT1b gangliosides. While it is still a matter of future research to determine the precise mechanism by which the two gangliosides contribute to the transcytosis of the G23-polymersomes, one possibility is foreseeable. The ganglioside GT1b is a natural receptor for the tetanus toxin, which attacks with high specificity motor neurons in human. Importantly, G23, which was previously identified and named Tet1, is like tetanus toxin transported in a retrograde mode to the higher order motor neurons in the CNS after injection in the sciatic nerve. Retrograde axonal transport of virus particles, which are of comparable size to the G23-polymersomes, is well documented and it is possible that G23-polymersomes are transported in a similar manner. Thus, it is of major interest to verify whether G23-polymersomes will be an efficient drug carrier with specificity for motor neurons after intramuscular administration, as such strategy will have an enormous impact on the treatment of amyotrophic lateral sclerosis or other neuropathologies with motor neuron origin. The G23 peptide may together with the RVG-9R peptide emerge as a new class of targeting vectors with dual function. On the one hand these vectors potentiate the transport of nano-sized particles across the blood-brain barrier, and on the other hand they target specific subpopulations of neurons, thus enabling enhanced bioavailability within distinct brain regions. While this might be a limitation in addressing disseminated brain tumors, it is a clear advantage in the prevailing CNS malfunctions, where only specific neurons are affected. Notably, in many instances of Parkinson’s disease, from two parallel neurons with dopamine innervation, usually only one is affected. This will impose a need for even more
stringent criteria to targeted therapeutic approaches. Another issue in successful drug delivery by means of targeted nanoformulations is the nature of the nanocarrier composition. Liposomes, prepared from natural lipids, are generally well-tolerated, and already provide a successful delivery platform for targeted therapy in many pathological conditions. However, liposomes may destabilize within the transport vesicles in the BBB endothelium and as a result release their contents before they reach their site of action. In case of the delivery of lipophilic drugs this may not be detrimental to the treatment as the drugs can diffuse from the endothelial cells further into the brain. However, for the delivery of biological macromolecules such as proteins, peptides, and nucleic acids, this would mean a dead-end. In contrast, polybutadiene-polyethyleneglycol polymersomes, as used in our studies, are non-biodegradable and remain intact throughout the complete transport process. The controlled disintegration of the polymersomes, which will allow for the release of a drug at the site of action, i.e. the brain tissue, is currently under investigation. Next to the G23-polymersomes, we showed plasma membrane vesicles, derived from neural stem cells, to efficiently accumulate in the brain parenchyma. Thus, PMVs represent a potentially attractive alternative delivery systems with major advantages: their natural origin and presumably lack of immunogenicity, and their apparent natural affinity for the brain. However, the harvesting of neural stem cells from the patient is not without risk. One possible solution, i.e., the re-programming of easy-to-harvest somatic cells such as fibroblasts into neural stem cells, was recently described. Clearly, bio-inspired nanoformulations belong to the future of targeted therapy.