Review

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Designing the future of nanomedicine: current barriers to targeted brain therapeutics

Abstract: Access of therapeutic agents to the central nervous system is generally restricted by an intricate barrier system, which protects the brain and maintains homeostasis. Endogenous nutrients, however, engage with the available molecular systems and successfully cross the blood-brain barrier (BBB). One of the systems, receptor-mediated transcytosis (RMT), involves non-stereoselective vesicular trafficking and has been extensively investigated as suitable for transportation of large molecular cargos. This review focuses on recent developments in elucidating the molecular pathways that regulate RMT and the fashion in which they impact the design of therapeutic nanosystems for BBB transcytosis. The challenges of brain targeting, however, are not restricted to successful engagement with BBB receptors, also including species-specificity, selectivity and maintaining targeting capability in real biological media.

Keywords: apoE; blood-brain barrier; drug targeting; nanoparticles; receptor-mediated transcytosis; transferrin.

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The delivery of many novel potentially therapeutic and diagnostic compounds to specific areas of the brain is restricted by brain barriers (1). Although the coexistence of three barrier layers (BBB, choroid plexus epithelium and arachnoid epithelium) confines and regulates the molecular exchange at the interfaces between the blood and the brain (2), the BBB offers the greatest control over the 20 m^2 of total surface area of the brain capillary endothelium, to create a microenvironment suitable for neuronal signalling and maintain central nervous system (CNS) homeostasis (3).

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The blood-brain barrier

The blood-brain barrier is a dynamically regulated and highly sophisticated barrier that keeps out harmful components and unwanted substances from the CNS, selectively allowing nutrients to reach the brain. The barrier is formed by cerebrovascular endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet linking to the neurons (4). Together with pericytes and microglial cells, they support the function and regulate the intercellular signalling to control the flow and trafficking to the brain (5, 6).

The unique features of the extremely selective permeability of the BBB are due to the specialized properties of the brain endothelium (7). Primarily, the tight cell-cell interactions lead to the formation of an intact physical barrier. The junctional complexes between adjacent endothelial cells are formed by tight junctions (TJs) and adherens junctions (AJs) (8). The TJs consist of complex structures of several transmembrane proteins such as occludin, claudins and junctional adhesion molecules (JAM), which are involved in sealing the barrier, regulating processes and providing additional support to the structure. Other cytoplasmic proteins such as zonula occludens (ZO-1, ZO-2, ZO-3) act as scaffolds linking TJs to the actin cytoskeleton, providing tightness and strength of the barrier (9). Moreover, the AJs constituted by transmembrane glycoproteins of the cadherin family, are involved in structural support and leukocyte trafficking. These junctions are dynamic structures that can be modulated by phosphorylation or dephosphorylation signalling (10). In addition, the endothelial cells are highly polarized conferring high electrical resistance to the barrier (11). Furthermore, a plethora of different integral membrane proteins,

including receptors (transferrin, insulin or LDL receptor), metabolic transporters (carrier-mediated transporters as glucose transporter, GLUT) and enzyme systems situated on the luminal and abluminal membranes, support the multiple functions of the BBB (12).

Transport across the BBB

The permeability and transport through the BBB is strictly regulated, capable to respond to physical, chemical or biological stimuli. Only small lipid-soluble molecules (with a molecular weight <400 Da) present the ability of passive diffusion through the membrane of the BBB (via the transcellular lipophilic pathway) without any metabolic energy requirements (13–15). Nevertheless, lipid in-soluble, polar molecules, small ions and macromolecules require different active processes to move across the BBB. Multiple endogeneous transport pathways, including tight junction relaxation (paracelullar pathway), energy dependent pathways, carrier mediated transport (CMT), efflux pumps and vesicular transport processes such endocytosis and transctytosis (receptor mediated transcytosis and adsorptive mediated transcytosis), allow the transport of these necessary biomolecules (ions, amino acids, glucose and other nutrients) across the brain capillary endothelium whilst preventing the entry of other molecules (Figure 1) (16–19).

Considerable efforts have been invested in discovering efficient strategies to transport therapeutic molecules to the brain as treatment for neurodegenerative diseases. Since more than 98% of the potential therapeutics are incapable of crossing the BBB (21), a better understanding of the natural endogenous processes to cross the barrier, including the identification of multiple pathways, key transport moieties and patterns, is needed to design and develop efficient drug delivery systems. Significant advances in the field of drug delivery have been carried out by implementing the tools afforded by nanotechnology (22). Nanoparticles offer multiple advantages, as theranostic agents and platforms to enable the study of the interactions between material surfaces and biological compounds involved in different mechanisms of transport (23).

Endogenous pathways, rather than disrupting cellular processes like opening tight junctions, are selected as transportation approaches to achieve this goal. The most facile approach would appear to be nanoparticle modification with lipophilic properties, introducing non-polar

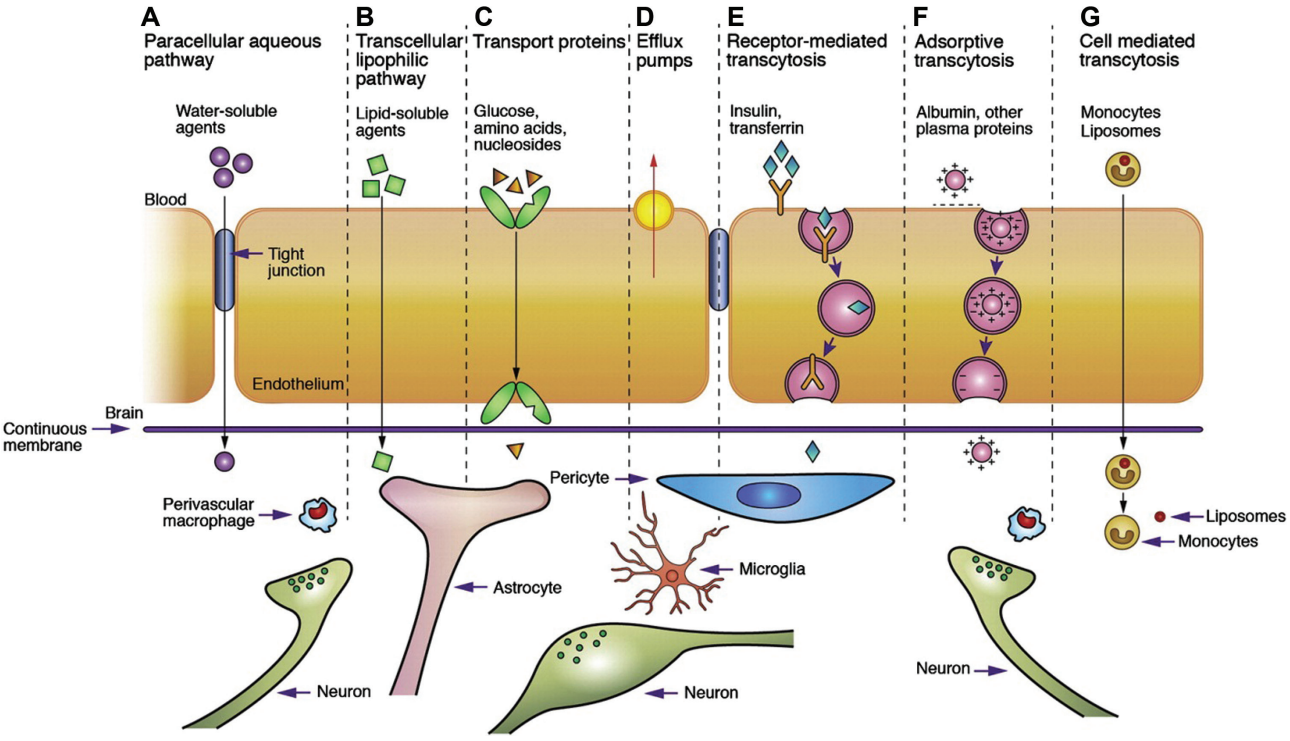

Figure 1 Schematic representation of transport pathways across the blood brain barrier (A) paracelluar pathway, (B) passive diffusion of lipid soluble molecules, (C) carrier mediated transport (CMT), (D) efflux pumps, (E) receptor mediated transcytosis (RMT), (F) adsorptive mediated transcytosis (AMT), and (G) cell mediated transcytosis. Reproduced with permission from reference (20).

moieties or molecules capable of passively diffusing the membrane (24). However these are prone to interact with efflux pumps, such as P-glycoprotein (P-gp), that protect the brain form the influence of many lipophilic compounds and do not offer an efficient approach for brain delivery (25).

Therefore, different targeting strategies have been explored for the transcytosis of nanoparticles and other therapeutic cargos through the BBB. Since large molecules and constructs do not penetrate the barrier, the strategy of engineering the so-called 'molecular Trojan horses' (MTH) has been employed as a method of delivering cargos into or past the barrier. A MTH system usually refers to a monoclonal antibody or an endogenous protein capable of crossing the BBB through a negotiation mechanism, fused to a therapeutic molecule (26). In principle this can be applied for any type of cargo (protein, nanoparticle), that succeeds in entering the brain from the blood through an endogenous BBB mechanism using a negotiation moiety. The overall aim is to insure that both the transport vector and the cargo maintain their functionality and integrity, to enable the successful recognition, and hence transport from the blood to the brain.

Among all the pathways that can be followed for successful brain delivery, the most promising candidates seem to be carrier-mediated transport (CMT), adsorptive-mediated transport (AMT), cell-mediated approach and receptor-mediated transcytosis (RMT).

In the case of CMT, nanoparticles need to be conjugated to endogenous compounds or analogues in order to permit a conformational change on the molecule resulting in the transport to the other side of the membrane. There are numerous systems available for endogenous molecules or essential nutrients: GLUT1 (Glucose Transporter 1) for glucose and mannose, amino acid transport systems for neurotransmitter precursors (neutral, acidic or basic), the monocarboxylic acid transport system for short-chain acids and the choline transport system etc. Recent attempts to quantify the protein expression levels of transporters by liquid chromatography–tandem mass spectrometry proved that the levels of CMT systems exceed the ones of receptors in the brain capillary endothelial cells (27, 28). Two of the carriers with high protein expression were EAAT1 (Excitatory Amino Acid Transporter 1) and GLUT1, both members of the solute carrier family (SLC). Their expression, measured in fmol/μg protein, was significantly higher than the one of transferrin receptor (TfR1) and low density lipoprotein receptor-related protein 1 (LRP1), two of the most abundant BBB receptors. While offering relevant quantitative information, this approach does not elucidate on which side of the barrier they predominate or their cellular location (capillary endothelium, capillary pericyte or astrocyte endfeet), both essential aspects for drug targeting (29).

Even though CMT systems present certain advantages for BBB endocytosis, such as large transport capacity and significant binding affinity, they are substrate and stereoselective, only engaging with endogenous or endogenous-like molecules (LAT1, one of the main transporters, specifically binds L-type amino acids). Furthermore, their trafficking capacity depends on plasma concentration of substrates and can easily reach saturation (30). Other restrictions to CMT include the affinity of the targeting moiety, which should be higher than the one of the substrate to avoid competition, and size of the cargo, transporters being less size-permissive than receptors. In spite of this limitation, a strategy to transport nano systems via CMT involved the design of a glucose-modified liposome to target GLUT1 and cross the BBB (31). By introducing a new ligand, the delivery system would engage with the luminal GLUT1 and avoid the undesired efflux from brain to blood mediated by abluminal GLUT1. However, one of the main limitations of using glucose as targeting moiety, in addition to its lack of specificity, is the disruption of glucose uptake in the brain, which might lead to severe cerebral damage (30) as GLUT1 is the only glucose transporter that supplies the brain.

The AMT pathway presents higher capacity but lower affinity due to its leading mechanism, of electrostatic interaction between the negatively charged membrane surfaces and the positively charged molecules (32). AMT-binding ligands, such as cationic albumin have have been reported as facilitating BBB negotiation of PEGylated nanoparticles (33). There is still significant knowledge required for establishing how selective these approaches (CMT, AMT, cell-mediated transport) are for BBB targeting and if they can in fact lead to transcytosis. In addition, little is known about their ability to drive the nanoparticles out of the endo-lysosomal pathway to avoid exposure to degrading enzymes and efflux pumps.

While the carrier-mediated transport of molecules is restricted by their size and stereospecificity, receptor-mediated endocytosis involves a more size-permissive, non stereoselective vesicular trafficking of various substrates (34). All the receptors expressed on the luminal and abluminal sides of the BBB contribute to brain homeostasis by transporting specific endogenous peptides in one (or both) directions: from blood to brain and from brain to blood. Receptor mediated endocytosis can transport large molecular weight compounds, via intracellular transport vesicles. This pathway, after ligand-binding to luminal surface

receptor and subsequent activation, leads to internalization via endosomes/lysosomes (endocytosis) or trafficking across the membrane to be externalized at the abluminal surface (transcytosis) (35). The major challenge of nanoparticle uptake by RMT consists of avoiding the lysosomal or ubiquitin-proteasome pathways to successfully achieve transcytosis to the abluminal side of the barrier.

Therefore, the simplest approach of crossing the BBB is by grafting endogenous polypeptides to nanoparticles and mimicking the natural exchange process: Transferrin for the Transferrin Receptor (TfR) targeting, and apolipoproteins for low density Lipoprotein Receptors (LDLR) (36, 37). The least feasible biomolecule for this is insulin (for the human insulin receptor, HIR), whose applicability in vivo is limited by the induction of potential glycaemic disorders.

Apolipoproteins as ligands

Among endogenous molecules with potential of crossing the barrier, an important class is that of substrates for the LDL receptors, expressed on the surface of the blood-brain barrier, neurons and glial cells (38). Apolipoproteins (apoE, AII, B, C, J) were investigated in the transport of nanoparticle-bound drugs to the brain (39, 40). These apolipoproteins were grafted to the surface of poly(butyl cyanoacrylate) nanoparticles with or without previous Tween 80 coating (polysorbates 20, 40, 60, 80 have been shown previously to facilitate apoE adsorption in human plasma) (41). The uptake of ApoE and ApoB-coated nanoparticles in brain endothelial cells was significant, most probably through receptor-mediated endocytosis and not driven by toxicity-related disruption or opening of the endothelial barrier, leading to diffusion or transcytosis to the brain parenchyma (42). The observed effects were reduced for nanoparticles without tween 80, most likely due to inhibition of P-glycoprotein by the surfactant, an efficient system that promotes the efflux to the luminal side of the BBB (43, 44).

For nanoparticles or drugs to reach the brain, the avoidance of efflux systems expressed in the brain endothelial cells is as crucial as insuring endocytosis. Members of the ATP-binding cassette (ABC) such as P-glycoprotein (Pgp/ABCB1) and multidrug resistance-related proteins (MRP1/ABCC1) contribute to the barrier function by transporting drugs back to the blood. Therefore, a recent approach aimed to deliver doxorubicin to brain tumours, expressing multidrug resistance properties, using ApoB100-coated nanoliposomes in association with statins as ABC inhibitors (45). Pre-treatment with simvastatin significantly increased the liposomal uptake and this was attributed to various mechanisms. Firstly, a pronounced and continuous inhibition of efflux transporters was recorded and attributed to increased production of NO and to a lesser extent due to decreased Pgp/ABCB1, MRP1/ABCC1 expression or glycosylation. Secondly, statins decreased the synthesis of endogenous cholesterol, leading to up-regulation of LDLR expression and subsequently, to increased receptor-mediated endocytosis of ApoB100-coated nanosystems. Interestingly though, the uptake of apo-liposomes without statins was not affected by free ApoB100 under basal conditions and was comparable to the control liposomes (no apolipoprotein), suggesting that the LDL receptors on the barrier surface (hCMEC/D3 cells) were not sufficient for receptor-mediated transcytosis.

This observation is in accordance with a previously investigated hypothesis, that LDLR are not abundantly expressed on the blood brain barrier, compared to other members of the LDL receptors superfamily (LDLRf), such as LDLR-related protein 1 (LRP1, scavenger receptors) and very low-density lipoprotein receptor (VLDLR), which might be more efficient targets for RMT (46). ApoB, a substrate for LDLR only, with negligible affinity to LRP1, needs therefore increased receptor expression for the efficient delivery to the brain. Taking into consideration that ApoE, however, is able to bind both LDLR and LRP1, a derivative of the receptor-binding domain of ApoE for the delivery of a lysosomal enzyme, α-L-iduronidase (IDUA), across the blood-brain barrier, has been engineered (47). Due to the implications of the natural, entire ApoE isoform in the pathways of neurodegeneration in Alzheimer's disease, peptides that conserved the ApoE receptor-binding region, without interfering with the endogenous ApoE-related processes, were screened in vitro (48, 49). This represented the first approach of creating a fusion protein containing an LDLRf binding domain of ApoE, resulting in seven IDUA-Rb fusion candidates being constructed and evaluated in a bovine brain capillary endothelial cell model and in vivo.

Low-density lipoprotein receptor-related protein (LRP) is as a highly expressed, multi-ligand receptor of the BBB cell surface. More than 30 different ligands, from different classes of molecules: apolipoproteins, growth and coagulation factors, chaperones, viral proteins etc. have been investigated so far, most of them binding to domains II and IV (50). The multifunctionality of LRP1 has rendered it an attractive target for nanoparticle brain delivery, with recent strategies using angiopep-2 as an LRP substrate. Angiopep-2 is a member of the angiopeps family of peptides, and was obtained from the amino acid sequence of aprotinin, a pancreatic trypsin inhibitor, aligned with

the Kunitz domain of human proteins (51). Brain delivery of anticancer PEGylated multiwalled carbon nanotubes (52), DNA-loaded nanoparticles (53) and conjugated poly(ethylene glycol)-co-poly(ε- caprolactone) nanoparticles has been reported as facilitated by angiopep-2 functionalization, through a clathrin- and calveolae-mediated endocytotic pathways. The process was found to be concentration, energy and time dependent (54).

While the efficiency of LRP1 in mediating endocytosis has been proven in numerous studies, its involvement in barrier crossing is still to be confirmed. Krieg and Herz hypothesized in 1994 that members of the LDL receptor family, including LRP, are mostly endocytic receptors, which generally promote lysosomal degradation of the substrate, as opposed to transcytosis (55). Investigations on Madin Darby Canine Kidney (MDCK) cells, analogous to BBB models, but of epithelial not endothelial origin, and brain microvascular endothelial cells (HCMEC/D3) cells aimed to determine the fate of an endogenous LRP1 ligand, amyloid-β protein (Aβ). In these models, LRP activity promoted the uptake of Aβ from the luminal membrane side, followed by degradation and not transcytosis of the intact peptide.

Even though these findings might be in accordance with the general hypothesis of lysosomal sorting induced by scavenger receptors, a recent study shows that LRP1 could promote transcytosis. By using two BBB in vitro models, wild type primary mouse brain capillary endothelial cells (pMBCECs) and pMBCECs from LRP1 knock-in mice with mutated LRP1 NPxYxxL endocytosis/sorting motif, genetic evidence for LRP1-mediated endocytosis and bidirectional transcytosis (luminal-abluminal) of Aβ without significant degradation was provided (56).

There is still significant research to be carried out to reach a thorough understanding of LRP1-controlled pathways in the BBB. Furthermore, similar to other scavenger receptors, these receptors are not selectively expressed on the brain endothelial cells, but also in macrophages and hepatic cells (57), where they exert important physiological function. For these reasons, the feasibility of using nanoparticles with moieties that bind scavenger receptors, for brain targeting, is still debatable. Ideally, nanosystems should be designed so as to selectively and efficiently exploit the BBB receptors' endocytic capacity without interfering with their biological function or undergoing lysosomal degradation.

Advances in TfR targeting

Another transporter that has been extensively studied for mediated BBB transcytosis is the transferrin receptor (TfR). Heterogeneously distributed in the cerebral tissue, TfR consists of two transmembrane subunits with extracellular domains, each binding one molecule of the endogenous Fe-transporting ligand, transferrin (Tf) (57). Due to the ability of internalizing large molecules and constructs, through a clathrin-mediated process, TfR can be used as a means of brain targeting by BBB transcytosis.

Coating nanoparticles with Tf for TfR engagement has been used extensively as a targeting strategy. In a recent study, gold nanoparticles of two different sizes, coated with variable amounts of transferrin were investigated to discern their ability to reach the brain parenchyma (58). The results show that both the nanoparticle size and amount of Tf decorating the surface was essential for the fate of these constructs and their capability to trigger different aspects of NP-TfR engagement. A low amount of protein led to a low affinity of the nanoparticles for the receptor with no internalization, (due to competition with endogenous Tf), while a high amount caused strong attachment to the endothelial cells, with no observable transcytosis. An optimal level of the Tf coating insured efficient interaction with the receptor, internalization and transport to the abluminal side of the barrier. This principle, of controlling the avidity of a construct for the TfR, for an optimized engagement in transcytosis was previously described (59).

By using an anti-TfR antibody and not Tf or Tf-like peptides, which can compete with Tf binding and disrupt essential biological processes, the approach aimed to identify whether the BBB negotiation of therapeutic agents can be enhanced by modulating the affinity of antibodies to TfR (59). For this purpose, high- and low-affinity anti-TfR antibodies were evaluated; findings suggested that brain uptake was enhanced for high-affinity anti-TfRs at low doses only, while at therapeutic doses low-affinity antibodies are significantly more efficient. Considering this inverse relationship between uptake and affinity, a bifunctional antibody, with an anti-enzyme b-secretase (BACE1) arm and a second low-affinity anti-TfR arm was designed (anti-TfR/BACE1) and evaluated in vitro and in vivo. The initial observations were confirmed and might be explained by the fact that, at sufficient doses, luminal TfR saturation does not depend on affinity. Moreover, a lower affinity triggers effective receptor-antibody dissociation upon endocytosis, leading to exocytosis to the brain side and not to lysosomal sorting. Finally, the presence of TfR receptors (and other efflux pumps) on the abluminal side, would easily contribute to inverse transcytosis when bound with high affinity or upon reaching saturating antibody concentrations in the brain (59).

Even though this proof of principle was a major advancement in brain targeting, the potency of the bifunctional antibody was limited. With the purpose of increasing transcytosis by receptor engagement and, therefore, pharmacological efficacy, two brain shuttles with monovalent and bivalent TfR binding were investigated (60). A single-chain (sc) Fab fragment of a monoclonal anti-TfR antibody was linked to one (single Fab construct, sFab) or both (double Fab construct, dFab) C-terminal ends of the heavy chain of a therapeutic monoclonal antibody (mAb31).

The Fab fragments were fused through a flexible glycine-serine linker and not by the general approach of using IgG-hinge regions for designing the bifunctional constructs (61–63). In addition to a more facile transportation due to lower molecular weight, Fab antibodies lacking the Fc fragment do not cause complement system-associated cytotoxicity and escape the Neonatal Fc Receptor-mediated recycling (FcRn) (64). Binding preferentially to the CH2-CH3 domain of IgG (65), in a pH-dependant process (66), FcRn is mostly localized in the intracellular compartment and, to a lower extent, on the abluminal side of the BBB and other endothelia (67). Its involvement in promoting transcytosis from brain to blood and exocytosis from the cells to the luminal side has led to strategies of avoiding engagement of brain targeting cargos to the FcRn. In addition to FcRn escape, the Brain shuttles bind to a distant domain of the TfR from that of endogenous Tf and therefore do not cause iron homeostasis disruption.

The affinities of the two constructs are different, sFab having a lower receptor affinity compared to dFab and, subsequently, a different fate. In vitro (brain endothelial cell lines, hCMEC/D3, bEnd3) and in vivo studies showed that dFab was rapidly internalized and either entered the lysosomal degradation pathway (>50%) or accumulated in the neurovasculature, without reaching the target. In addition, dFab-TfR interaction leads to significant down-regulation of the receptor, not by transcriptional regulation of TfR expression, but by impeding its recycling back to the luminal surface. These assumptions were re-confirmed recently by total internal reflection fluorescence microscopy tracking at a single molecule level, of endogenous TfR, labelled with anti- murine TfR Fab-quantum dots (Tf R-Fab:QD) (68). Tf R-Fab: QDs recognize a different epitope from that of anti-TfR constructs or Tf and do not induce lysosomal degradation. This can be attributed to a conformational change and dimerization that dFab causes in the structure of TfR upon binding, which leads to lysosomal signalling or receptor ubiquitination and, subsequently, removal from the recycling pathway and destruction (60, 69).

The sFab construct, however, undergoes clathrin-mediated endocytosis and successful transcytosis to the brain parenchyma (Figure 2). The mechanism by which sFab manages to escape lysosomal sorting is still unclear. It most likely induces no change in TfR recognition along the endosomal pathway, hence no alteration in the physiological recycling process.

Therefore, it is important to elucidate how affinity or valence influences RMT and intracellular sorting in order to optimize the strategy of brain targeting through BBB crossing (70). A recent in vitro assay advanced the

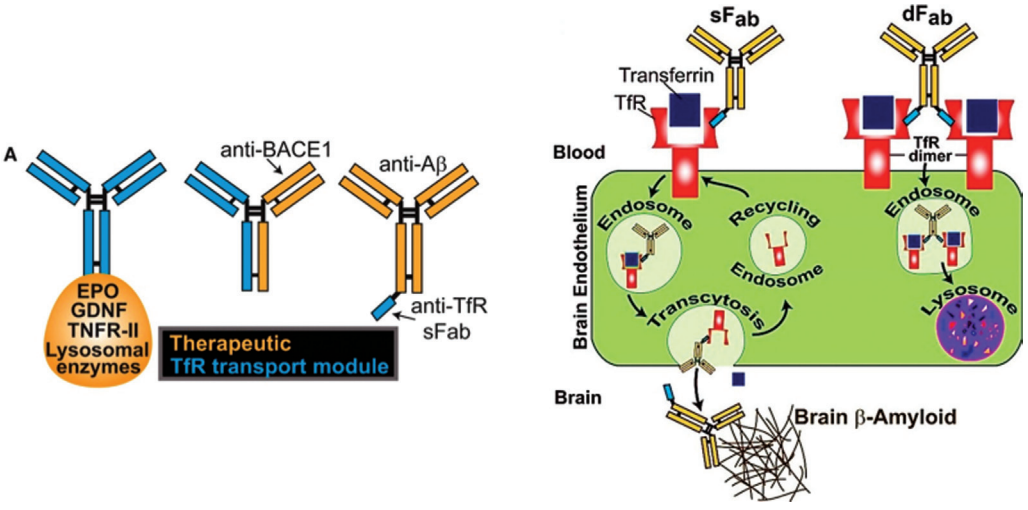

Figure 2 Novel RMT strategy based on the antibody-Tf receptor affinity modulation: Designing of modular anti-transferrin receptor Fab shuttles to cross the BBB. Lower Tf receptor affinity of the construct leads to exocytosis to the brain side, avoiding lysosomal pathway. Reproduced with permission from reference (70).

understanding of this process by correlating the transcytosis capacity of anti-TfR antibodies with pH-dependence of the receptor-antibody binding (71). While intrinsic affinity may have an influence on transcytosis, antibodies with similar affinities do not necessarily undergo the same pathway. Specifically, antibodies with lower affinity for TfR at acidic, late endosomal pH, are the ones most prone to escape degradation. Those with high affinities and bivalent binding are irreversibly cross-linked to the receptor and impede its recycling. It must be mentioned, however, that this mechanism has only been explored in one cell line so far, hCMEC/D3, with further studies being required for validation.

A less explored pathway: diphtheria toxin receptor

Less explored than other endocytic systems, the diphtheria toxin receptor (DTR) triggered attention due to some of the advantages it offers over other receptors. DTR is a membrane-bound, BBB, glial cells and neurons-located precursor of heparin-binding epidermal growth factor (HB-EGF) (72). It has no endogenous ligands, therefore targeting DTR is neither competitive nor interfering with homeostatic processes.

Brain targeting via DTR interaction has been employed by using cross-reacting material 197 (CRM197), a diphtheria toxin mutant with no toxicity or immunogenicity. This currently marketed carrier was investigated in vitro and in vivo for the proof-of-principle (BBB transcytosis by RMT) (72) and for its capacity to traffic large constructs, such as nano liposomes (73) or polybutylcyanoacrylate nanoparticles (74). Studies were performed on different cell lines and led to inconsistent results, as CRM197-grafted nanoparticles crossed human brain-microvascular endothelial cells (HBMECs) (74), while CRM197- modified liposomes displayed only binding without transcytosis of murine endothelial cells (bEnd.3) and no targeting in vivo (75).

From design to efficacy: the challenges

Unquestionably, significant advancements have been made in the field of crossing the BBB. Since mechanistic studies are essential for efficient targeting, elucidation of certain pathways regulating endocytosis and transcytosis will contribute to the intelligent design of medicines.

However, there are still many challenges to overcome in order to develop a safe, clinically relevant brain delivery platform. Essential to this is the species-specificity of currently studied antibodies: a rat mAb, OX26, to the mouse TfR is not functional as a Trojan horse for therapeutic delivery in a mouse model, while two engineered rat monoclonal antibodies, 8D3 and RI7-217, to the mouse TfR succeeded in transcytosing the BBB (76). A murine mAb to the human insulin receptor (HIR) can be tested in rats, but not in humans due to immunological response to a mouse antibody (77). For these approaches to be applicable in human studies, chimeric and humanized antibodies should be engineered and evaluated.

Another aspect is the expression of most of the BBB receptors in peripheral tissues. This raises the concern of targeting specificity and interference with physiological pathways. TfR, for example, is also present in the liver, bone marrow (57) and reticulocytes and these receptor populations should not be affected by BBB-TfR targeting agents. Using PEGylated nanoparticles to avoid liver clearance and increase circulation time (78), while useful for the avoidance of hepatic receptors, does not solve the issue of receptors that are expressed and regulate the iron homeostasis of blood cells. The studies of bispecific transferrin receptor (TfR)/β-secretase (BACE) antibodies were performed on mice and showed significantly increased brain uptake. These initial findings were advanced by studying the effect of affinity modulation on peripheral exposure and was found that reducing TfR affinity of bispecific antibodies, unlike traditional monospecific antibodies, decreases peripheral exposure in the favour of BBB exposure (79).

Since the initial PK/PD (drug exposure vs. therapeutic activity) profile was promising, the next issue to be addressed was safety profile upon chronic administration. As mice reticulocytes have a high density of TfR, severe reticulocyte reduction was observed from the first dose, which raised a major toxicity concern. The undesired effects were significantly lowered after engineering antibodies with no Fc interaction with immune cells (Fc leads to macrophage and monocyte activation, subsequently causing reticulocyte destruction) or interference with the complement cascade, two of the main toxicity mechanisms. Even though these observations might raise concerns regarding clinical applicability, translation from rodents to primates and humans might actually be promising, since red blood cells were found to mature in the bone marrow (where antibodies do not have access) in humans and not require TfR-mediated iron supplies from the blood for this process (79). This is a major step forward in designing antibodies with boosted brain uptake, however

for clinical studies antibodies against human receptors are required, which would subsequently reiterate the long process of distribution, efficiency and toxicity evaluation.

Similarly, HIR, another receptor investigated for BBB transcytosis might lead to severe physiological effects upon binding of targeted systems, such as those observed in monkeys treated with a HIRmAb-construct (80). Additionally, HIR is highly expressed and regulates glycaemic levels in peripheral tissues, rendering any attempt of selective brain delivery as extremely difficult and with severe consequences. An approach that has been investigated is the administration of therapeutic systems via the intra nasal route. Even though not a targeting strategy, the intra nasal pathway avoids interaction with peripheral receptors and transporters and can be extended to other systems or transcytosis pathways (delivery of peptides to the brain to avoid lymph nodes and splenic loss) (81).

Another core aspect that should be considered when designing targeted therapeutics, including antibody constructs or grafted nanoparticles, is their behaviour in biological media. It is clear that in vitro efficacy does not translate to in vivo potency (as illustrated by poor pharmacokinetics of brain delivery systems) and this is also due to the functional changes that systems undergo upon exposure to relevant biological fluids.

Targeting in biological fluids

Biological fluids, by their nature, are incredibly complex containing all the essential requirements for cells and organs to function. In particular human blood plasma is exceptionally intricate consisting of ca. 4000 different biological components including proteins, peptides, lipids, salts and small molecules. Moreover, the range of concentrations spans over 12 orders of magnitude with some of the low abundance proteins playing key biological roles (82). When nanoparticles are exposed to such complex conditions their surface is drastically altered, within a short time frame the biomolecules present in the milieu adsorb on the surface to lower the surface energy, forming a corona of biomolecules (83, 84).

The biomolecular corona is a multi-layered interface characterised by two regimes segregated by the difference in their affinity for the particle surface and thus their residence time. The ‘hard’ corona consists of biomolecules which reside on the particles surface for sufficiently long time periods, while the ‘soft’ corona includes all the other biomolecules which interact with the nanoparticle, be it through long range particle interactions or protein-protein interactions. This second layer is dynamic and the biomolecules present therein are under constant flux, exchanging between the surface and the rest of solution, and thus evolves over time (85, 86).

This biological coating confers a new identity to the nanomaterial (87, 88), changing the way that cells and biological machinery interact with it (Figure 3). Mitigating or controlling the formation of the biomolecular corona is therefore a crucial design element of any system for medical applications. One method is to mitigate the adsorption of biomolecules to the surface using the antifouling properties of small hydrophilic polymers e.g., poly(ethylene) glycol (PEG), to reduce the binding of proteins and thus the formation of the corona. It has been shown that these polymer layers can confer ‘stealth’ properties masking the nanoparticles from the immune system (89).

Another approach is to use nanoparticles with controlled surface properties to preferentially adsorb selective proteins from the biological milieu. In one study Zensi et al. used a non-ionic surfactant to cross the blood brain barrier (90). In another study the surface of nanoparticles was modified to selectively bind melittin, a toxin found in bee stings. Using a molecular imprinting approach, in which monomers are polymerised in the presence of the target biomolecule, it was possible to create the correct surface chemistry and topology for optimum binding of the target species. This approach was then verified in an in vivo murine model by using these particles to successfully neutralise the toxin post administration (91). This approach could be adapted to selectively harvest biomolecules directly from blood plasma, in situ, which are implicated in BBB negotiation and transcytosis, providing a versatile approach for BBB targeting.

Design of nanomaterials for targeted diagnostics and therapeutics is an area of great importance. The concept of delivering therapeutic payloads in a controlled fashion to only the cells of interest is exceptionally attractive. Recently it has been shown that not all design strategies, which retain function under in vitro conditions, will successfully target under an in vivo setting. In particular it was shown that silica particles which had been modified with a PEG layer and subsequently transferrin protein were able to target the transferrin receptor pathway in idealised in vitro conditions, however in high serum concentrations this interaction between receptor and ligand was compromised, resulting in a loss of particle functionality (92).

It is not always the case that the particles lose targeting efficiency in blood plasma. In a recent collaboration by the same authors, using a similar nanoparticle design, single domain antibody conjugated silica nanoparticles retained their functionality in relevant biological

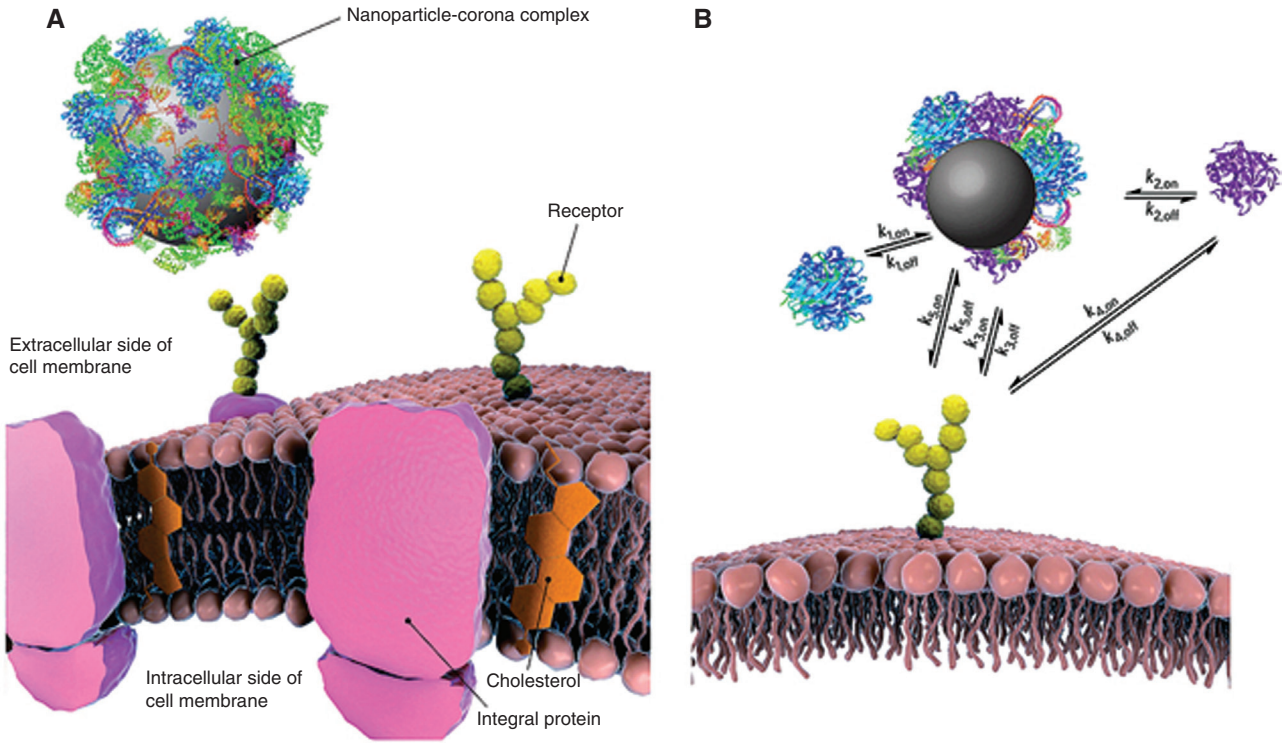

Figure 3 Biological identity of nanomaterials (A) biomolecular corona formation determines the uptake, transport and fate of nanoparticles in living systems; (B)Nanoparticle – (hard and soft) protein corona complex formation under biological environment exposure. Reproduced with permission from reference (83).

environments (93). Whilst in the previous study the specificity of uptake of transferrin grafted nanoparticles was completely lost in serum containing medium, the single domain antibody conjugated constructs still displayed discriminatory uptake through the targeted pathway at high serum concentrations.

From these studies it is clear that the choice of ligand-receptor complex is a key design element when constructing nanoparticles whose intended application brings them into contact with biological fluids, in particular blood. It highlights the contrast between in vitro and in vivo testing conditions and how particle functionality can be reduced or even lost in realistic biological environments. It also demonstrates the importance of the correct in vitro testing conditions when estimating the targeting efficacy prior to in vivo testing. Moreover the contrast between these two studies demonstrates that more knowledge is required on the mechanisms behind the loss of targeting in biological fluids.

In conclusion, current knowledge describing the parameters which govern the interaction of targeted nanomaterials is not clear and consistent. The challenges associated with creating constructs to effectively pass the BBB are numerous and strategies to circumvent these are not always straightforward. While receptor-mediated transcytosis is a fundamental pathway for BBB negotiation, systematic, mechanistic, studies are required for thorough understanding of molecular pathways, efficient design of targeting moieties and nanosystems, behaviour in biological media and pharmacokinetic and safety profiles.

Even though recent developments in antibody engineering have improved the knowledge on brain therapeutics, by addressing issues such as increased targeting and avoidance of peripheral loss through affinity modulation, there are still significant efforts to be invested for translation from research to clinical applications.

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