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The Roads We Take: Cellular Targets and Pathways Leading Biologics Across the Blood–Brain Barrier

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Biologics are at the frontier of CNS disease treatment. This applies both to therapeutic molecules such as peptides, antibodies and RNA interference agents, and to delivery vehicles of biological origin such as viral vectors and extracellular vesicles. Unlike small molecules, biologics are not likely to diffuse across cell membranes. To get into and across brain capillary endothelial cells (BCEC) forming the blood–brain barrier, they normally employ active, energy-dependent processes. They can initiate these processes non-specifically or trigger them by interaction with various receptor or transporter molecules at the luminal surface of BCEC. Designing biologics to use this specific engagement is more common in smaller formats, especially peptides and antibodies, but can also apply to targeted vehicles. This targeted design has employed a number of molecules expressed on BCEC – the transferrin receptor being the most common example, although there has been progress in identifying molecules that are even more specific to BCEC. In addition, the format of biologics and a multitude of their biophysical properties affect the way they interact with BCEC, and this diversity is even more salient between different classes of biologics. It affects the entire span of interaction with BCEC, from the initial engagement at the luminal surface to intracellular sorting, and eventually, entrapment or routing toward exocytosis into the brain parenchyma. In this article, I reviewed the progress in identifying novel targets that make the interactions between biologics and BCEC more specific, and in our understanding of the interplay between the properties of biologics and these interactions.

Keywords: blood–brain barrier, biologics, endocytosis, receptor-mediated transcytosis, intracellular transport, targeted drug delivery

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INTRODUCTION

Brain targeting is needed for intravenously (IV) administered biologics because otherwise their brain accumulation after an IV injection is low. The statement above is an oversimplification on several counts. First, 'low' is relative: for instance, while ~0.1% of the injected dose is indicated for monoclonal antibodies (mAbs) (Bard et al., 2000; Boado et al., 2010; Atwal et al., 2011), the relevant question is how much of the therapeutic needs to reach brain parenchyma to achieve a sufficient effect, and, consequently, whether the safety profile allows reaching that level. Second, the site of entry may well be crucial: a fraction of a biologic reaching the brain across the blood–brain barrier (BBB) may have a different distribution and, consequently, effect than the same fraction entering the brain from the choroid plexus, owing to the high density of the capillary bed with small (~25 μm) distance to the nearest cells such as neurons (Schlageter et al., 1999; Mabuchi et al., 2005).

Third, unmodified large biologics serving as delivery vehicles—e.g., adeno-associated viruses (AAVs) (Foust et al., 2009) or extracellular vesicles (EVs) (Wiklander et al., 2015; Banks et al., 2020)—have been shown to reach the brain; although the extent of this accumulation varies considerably, the percentage of the injected dose also has a different meaning in this scenario, at least for EVs because of their large loading capacity.

Nevertheless, targeting has been shown to increase brain transport, and therefore is an attractive option at least for that reason alone, and, possibly, for getting access to a greater brain volume. Here, I focus on several aspects: 1) interactions of non-targeted large biologics, 2) strategies for choosing targets and optimizing interactions with them, 3) considerations relevant for different formats of biologics, and 4) transport mechanisms helping them cross the BBB.

NON-TARGETED LARGE BIOLOGICS

If a vehicle is not intentionally targeted to certain molecules on brain capillary endothelial cells (BCEC), this does not necessarily mean that its interaction with BCEC is not specific. An early example comes from nanomedicine, where the interaction of nanoparticles (NPs) with BCEC is thought to be at least partially mediated by apolipoprotein E (ApoE) recruited in the bloodstream as part of the protein corona and subsequently interacting with the low-density lipoprotein receptor (LDLR) on the surface of BCEC (Kreuter et al., 2002). The point here is not so much ApoE per se but the fact that the recruitment of proteins from the bloodstream can mediate specific interactions with cellular receptors in hepatic or extra-hepatic delivery (Akinc et al., 2010; Dilliard et al., 2021). It seems reasonable to expect similar mechanisms applied to EVs, where not only biophysical properties, but also the protein signature on the EV surface can affect corona formation—or, indeed, itself mediate specific interactions with BCEC (Haqqani et al., 2013; Qu et al., 2018). Another example comes from AAV studies where the transport of PHP.eB capsid across the BBB was found to be mediated by lymphocyte antigen 6 complex, locus A (Ly6a)—notably, explaining the lack of PHP.eB transport in other species that do not express Ly6a, and, partially, in other mouse strains which have a reduced amount or an altered genetic variant of it (Hordeaux et al., 2018; Huang et al., 2019; Mathiesen et al., 2020). These observations point that non-targeted biologics, especially in large formats, may turn out to engage specific targets—or their combination—on the surface of BCEC, owing to their intrinsic properties or the properties they acquire in the bloodstream. Furthermore, the complexity of vehicles can make them likely to engage several extracellular and cellular actors spanning the entire range from the glycocalyx to the plasma membrane and intracellular sorting, to exocytosis on the abluminal side. AAV field again offers an example, with cellular interactions of AAV9 mediated by at least three different receptors, terminal β-galactose on glycocalyx, laminin receptor, and adeno-associated virus receptor (Akache et al., 2006; Shen et al., 2011; Pillay et al., 2017). This essentially redefines targeting, shifting it from the luminal membrane of BCEC to all cellular interactions, where individual steps of internalization, sorting, and exocytosis can be more or less specific to BCEC. It is unclear whether this level of specificity can be designed rationally. However, large-scale screening, common for AAVs and, more recently, lipid NPs (whose lessons can, to some extent, apply to EVs) (Akinc et al., 2008; Whitehead et al., 2014; Dahlman et al., 2017) can provide insights by generating BBB-penetrant vehicles (e.g., AAV capsids PHP.B, PHP.eB, AAV.F, CAP-B10, CAP-B22, CAP-Mac, 9P801, etc.) (Deverman et al., 2016; Hanlon et al., 2019; Nonnenmacher et al., 2021; Chuapoco et al., 2022; Goertsen et al., 2022) but also by identifying patterns promoting accumulation in different organs, including the brain, at least within a given class. This data, especially if available for different species, could then inform a computational design (Ogden et al., 2019; Bryant et al., 2021).

TARGETS AND TARGETED BIOLOGICS

I will focus on optimizing biologics for targeted delivery, the choice and characterization of targets, and the possible benefits of combinatorial targeting, using the transferrin receptor (TfR) and low-density lipoprotein receptor-1 (LRP-1) to illustrate the first two aspects. A detailed account of other commonly used BCEC targets and their use in delivery across BBB can be found in comprehensive reviews elsewhere (Lajoie and Shusta 2015; Terstappen et al., 2021).

Since the discovery of the TfR expression on BCEC (Jefferies et al., 1984), and the initial attempts to use it as a molecular Trojan horse (Friden et al., 1991; Friden et al., 1993; Huwyler et al., 1996), TfR has remained among the most studied shuttle targets. It has been used to enhance the transport of a broad range of molecules and vehicles, including peptides, mAbs, EVs, or even AAVs (Friden et al., 1993; Wu and Pardridge 1998; Yu et al., 2011; Zhang et al., 2018; Crook et al., 2020; Kim et al., 2020). The rationale for using TfR would be its high expression on BCEC and specificity to BCEC compared to other endothelial cells (Jefferies et al., 1984), although this specificity is limited as TfR is also highly expressed on certain cells of non-endothelial origin, including, notably, reticulocytes (Jandl and Katz 1963; Couch et al., 2013; Sun et al., 2019). A TfR-targeted biologic is also the only currently approved large molecule exploiting targeted delivery across the BBB—pabinafusp alfa, approved in Japan for the treatment of mucopolysaccharidosis type II, comprises iduronate-2-sulfatase fused to a high-affinity, bivalent TfR mAb (Giugliani et al., 2021). TfR-mediated delivery optimization has taken much effort, focusing primarily on antibodies or their fragments enabling the transport of payloads or large vehicles across the BBB. Insights derived from this optimization are likely relevant for other targets, although, ultimately, every biologic-target complex may have its unique signature affecting BBB transport.

Early studies using TfR mAbs employed them in a bivalent format, with the mAb fused to the therapeutic payload or decorating larger vehicles which could themselves contain the payload. While there is little doubt that this allowed TfR-targeted

constructs to internalize in BCEC, one key factor in the interpretation of these studies is whether the constructs would leave BCEC to enter brain parenchyma. Capillary depletion studies have shown that bivalent TfR constructs remained largely restricted to brain endothelium (Moos and Morgan 2001). Subsequently, (Yu et al., 2011) improved brain transport by reducing the affinity to TfR in a bispecific mAb with one arm targeting TfR and the other β-secretase (BACE1), while (Niewoehner et al., 2014) refined the optimization from a different angle, improving the transport of a bivalent anti-amyloid β mAb by fusing a Fab fragment of an anti-TfR mAb to its heavy chain, thus enforcing monovalent binding to TfR. Clearly, as far as TfR engagement is concerned, the approaches taken in (Yu et al., 2011) and (Niewoehner et al., 2014) are not entirely dissimilar since a bispecific antibody with one arm targeting TfR is essentially a monovalent shuttle. An interesting attempt to combine the bivalent format with the monovalent binding mode was made in (Hultqvist et al., 2017), where a bivalent TfR mAb was forced to engage TfR as a monovalent binder by a linker sterically preventing bivalent binding. Further optimization included the use of single-domain antibodies with low (Wouters et al., 2020) or high (Stocki et al., 2021) affinities, the use of a pH-dependent binder (Sade et al., 2014), and a variation of the monovalent, low-affinity binding approach with the binder engineered into the Fc domain of therapeutic mAbs (Kariolis et al., 2020; Arguello et al., 2022). This optimization also informed the design of NPs decorated with TfR mAbs for brain penetration—by simply using optimized TfR mAb fragments on the surface of NPs (Johnsen et al., 2018) but also, in a manner distinct to large particles, fine-tuning the density of those fragments on the particle surface, thus modifying the particle's avidity (Wiley et al., 2013; Johnsen et al., 2019).

LRP-1 is another well-explored BCEC target. A family of Kunitz domain-derived peptides named Angiopeps, particularly Angiopep-2, has been shown to cross BCEC (Demeule et al., 2008a) through an LRP-1–mediated mechanism (Demeule et al., 2008b; Bertrand et al., 2010). In (Sakamoto et al., 2017), an Angiopeps family-unrelated peptide identified in phage display and named L57 was shown to bind LRP-1 and accumulate in the brain after an IV injection. LRP-1 has been used to enhance NP-mediated transport of biologics across the BBB (Ke et al., 2009; Tian et al., 2015), with one study optimizing the LRP1-mediated transport of NPs by tuning the avidity to the receptor (Tian et al., 2020), which parallels the approach taken by Wiley et al. (2013) for TfR-mediated NP transport. Additionally, ANG1005, comprising three paclitaxel molecules covalently linked to Angiopep-2, has been used in a clinical trial for the treatment of recurrent brain metastases from breast cancer (Kumthekar et al., 2020).

The key question with LRP-1-mediated transport is probably whether LRP-1 exists in *in vivo* BCEC in the first place. Single-cell transcriptomics data suggest that it may not (Yang et al., 2022; Chen et al., 2020). Proteomics data may seem more encouraging, with several reports indicating LRP-1 presence (Uchida et al., 2011; Al Feteisi et al., 2018; Al-Majdoub et al., 2019; Campeau et al., 2020), although, notably Zuchero et al. (2016) did not find LRP-1 in a proteomic characterization of BCEC after $CD31^+$/$CD45^-$ fluorescence-activated cell sorting. One caveat with proteomics studies is the contamination with cells of the brain parenchyma (Al Feteisi et al., 2018; Al-Majdoub et al., 2019). The presence of e.g., aquaporin-4 or glial fibrillary acidic protein could be a clear sign of this contamination, and these markers are in fact present in untargeted proteomic studies (Al Feteisi et al., 2018; Campeau et al., 2020). The fact that LRP-1 is highly expressed in parenchymal cells (Zhang et al., 2014; Munji et al., 2019) further complicates things—one would expect contamination to be less of an issue for proteins with negligible gene expression on those cells, but in the case of LRP-1, their contribution may well be substantial. In addition to specific cell sorting, laser-capture microdissection (LCM) with sufficiently small sections could shed light here since sections with no trace of exclusive parenchymal cell markers and with a strong presence of e.g., claudin-5 could be reasonably expected to include only whole BCEC or their lumen-facing fractions, and in those sections, the presence of LRP-1 or lack thereof would be definitive. Spatially-resolved proteome of the brain cortex was studied using LCM with small sections in Zhu et al. (2018). However, while the proteome of 100 μm and especially 200 μm sections in Zhu et al. (2018) appears sufficiently rich within the coverage limit of the study, it also shows a strong presence of parenchymal markers, while the proteome of the smallest sections (50 μm) does not seem to provide a conclusive answer, especially given the small sample size. Ultimately, the question of a target's presence and level on BCEC, whether for LRP-1 or in other cases where a target's expression on BCEC is uncertain, will likely be addressed by single-cell proteomics, assuming sufficient coverage.

Combinatorial targeting can, in principle, increase the probability of circulating biologics' interaction with BCEC. This approach is likely easier to implement for larger vehicles such as NPs and EVs, than for mAbs and especially for peptides. Dual and even triple targeting to BCEC has been shown to increase the transport of liposomes and niosomes across the BBB (Markoutsa et al., 2014; Mészáros et al., 2018; Veszelka et al., 2021). One relevant aspect here could be the purpose of the second—or subsequent—moieties. If all shuttle moieties are present simply to increase the specificity of interaction with BCEC or, alternatively, if all are optimized for transporting the vehicle across BCEC, this may be moot. However, if all but one of them are present to increase the retention on the surface of BCEC, increasing the probability that the primary, optimized moiety will interact with its intended target, then, conceivably, this approach could benefit from targeting molecules in close proximity to each other. As an example, solute carrier family 7 member 5 (SLC7A5) forms a heterodimer with the CD98 heavy chain (CD98hc) (Lee et al., 2019); this and similar combinations of spatially close targets could potentially be exploited for a greater efficiency of interaction with BCEC.

Overall, the progress in single-cell studies and the availability of proteomics data have fostered the discovery of new potential targets at BCEC (Shusta 2005; Mäger et al., 2017). In a recent study, Cegarra et al. (2022) explored integral membrane protein 2A (ITM2A) as a potential shuttle target at the BBB identified by the Collaboration on the Optimization of Macromolecular

Pharmaceutical Access to Cellular Targets consortium; while the study results are not encouraging for the use of ITM2A, it is likely that large-scale omics-based characterization efforts such as those undertaken by the Human Cell Atlas (Regev et al., 2017; Eraslan et al., 2022) will be used to explore new targets allowing improved transport across the BBB.

Formats

There are several considerations affecting biologics, some more relevant to certain classes. The transport of naked oligonucleotides across the BBB is negligible, disfavored not only by a poor half-life but also by electrostatic repulsion by the negatively charged BCEC glycocalyx abundant in sialic acid residues (Vorbrodt 1989). mAbs benefit from neonatal Fc receptor-mediated recycling, increasing their circulation time in comparison to other types of biologics (Ghetie et al., 1996). While pharmacokinetic considerations are out of the paper's scope, one key factor linking them to cellular interactions is whether the reduced affinity to BCEC targets, which potentially increases dissociation from the target and transport into brain parenchyma, is of greatest benefit to mAbs precisely because of the longer half-life giving them more time to interact with BCEC specifically, thus compensating for the reduced binding to BCEC targets. This would clearly affect e.g., peptides and nanocarriers decorated with targeted ligands. However, a study of gold NPs decorated with low-affinity or monovalent TfR-targeted mAbs indicates that this benefit extends to NPs as well, despite their shorter half-life (Johnsen et al., 2018). Furthermore, the larger size of nanocarriers may adversely affect the extent to which they get across the BBB, penetrate the narrow-spaced barrier formed by astrocytic endfeet beyond the BBB (Kucharz et al., 2021), and distribute in the brain parenchyma with the extracellular space pore size estimated at ~40–65 nm (Thorne and Nicholson 2006). This can be offset by the substantial loading capacity of nanocarriers, allowing the release of many payload molecules for a single transcytosis event. The density of the targeting ligands on the surface of nanocarriers further allows transport optimization (Wiley et al., 2013; Johnsen et al., 2019). Protein corona formation is another factor specific to nanocarriers: in addition to modifying the intrinsic properties of nanocarriers in the bloodstream, it can also mask targeting ligands, thus precluding their specific interaction with BCEC (Salvati et al., 2013; Xiao et al., 2021). A way to address this limitation by employing a defined, pre-formed corona has been proposed in Kaleta et al. (2020).

TRANSPORT MECHANISMS

With TfR targeting, one attractive rationale has been that targeted biologics would simply follow the presumed route taken by the native ligand (Bien-Ly et al., 2014; Niewoehner et al., 2014). In this scenario, a biologic-target complex would proceed from the luminal to the abluminal side of BEC to release the payload—as, presumably, would Tf with TfR. This reasoning could work for TfR and several other receptors such as LDLR, known or thought to carry native ligands across the BBB. It is less clear for other targets. For instance, Zuchero et al. (2016) identified and explored basigin, glucose transporter 1 (GLUT1), and CD98hc as promising targets for mAb transport, demonstrating brain accumulation and the therapeutic effect of a CD98hc/BACE1 bispecific. Leaving basigin with its receptor and chaperone functions aside (for its further exploration as a BCEC target see (Christensen et al., 2021)), one cannot but wonder what would be the mechanism of trans-BBB transport in the case of mAbs or other biologics targeting CD98hc and GLUT1 (SLC3A2 and SLC2A1, respectively). The native transport mechanism invoked by solute carriers (SLCs) is facilitated diffusion, i.e., they engage no endocytic machinery behind the luminal surface of BCEC as far as their postulated function is concerned. Additionally, the size of most, if not all biologics is beyond that of molecules natively transported by SLCs. As a result, it may not seem likely that biologics engage the native transport mechanism of SLCs. However, if one excludes this native mechanism, it is conceivable that biologics' engagement with CD98hc and GLUT1 itself induces endocytosis after the binding event or simply exploits the constitutive recycling process of SLCs, in which case protein turnover becomes highly relevant. Then, the question would be how the biologic would get to the abluminal side. One possible explanation is that the complex, or only the biologic that is part of it, is artificially redirected toward exocytosis, owing to the biologic's properties that would enable such redirection.

Notably, in the case of TfR targeting, it has been postulated that a suboptimal format can fully or partially re-route a biologic toward lysosomal degradation in contrast to Tf and TfR that presumably reach the abluminal side natively (Pardridge et al., 1991; Bien-Ly et al., 2014; Niewoehner et al., 2014). One then has to wonder: why, conversely, would an optimal format not be able to re-route a biologic, with or without the target, to the abluminal side, even if the native ligand and/or target molecule do not normally reach it? Taking that reasoning further and applying it to the TfR-mediated transport, the fundamental question would be: does TfR get across the BCEC at all, in any scenario? This discussion would have two distinctly separate components: the fate of the native TfR–Tf complex, and the fate of TfR in a complex with a biologic that crosses the BBB. Both components are out of scope for this paper; for an overview of the former, focusing on the insights from the iron metabolism field, see (Skjørringe T et al., 2015; Duck and Connor 2016).

All these considerations may be more fundamental than practical in nature; after all, targets thought to be expressed on and, better yet, specific to BCEC have been exploited for brain delivery regardless of the tentative underlying mechanism. However, they may inform the strategy behind choosing targets and optimizing interactions with them. In this scenario, the early endosome likely becomes the nexus defining the fate of a biologic inside BCEC, and the desired outcome, in most cases, would be to route the biologic from that nexus toward the abluminal side. Ultimately, one key factor governing this (re)direction is likely the entirety of the properties characterizing the target–biologic complex or the biologic alone, depending on what is destined for exocytosis. Intuitively, key properties can include conformation and size,

the latter being far easier to optimize than the former. In addition to size, optimization could also be focused on the probability of the biologic's dissociation from the target. For instance, one could argue that the efforts to optimize TfR-mediated transport, described above, converge on two themes: the size of the biologic–TfR complex and the probability of the biologic's dissociation from TfR along the endocytic pathway or on the abluminal side of BCEC. The monovalent binding mode presumably improves the transport by eliminating crosslinking of TfR by two arms of the mAb (Niewoehner et al., 2014; Hultqvist et al., 2017). The actual observation is that the cross-linked complex is prone to lysosomal routing and eventual degradation. The question, however, is why; and one possible explanation is that the cross-linked complex is too large for any other intracellular fate, whether recycling to the luminal membrane or routing to the abluminal side. The same considerations are clearly applicable to bispecific mAbs. In a different vein, changing antibody affinity and making its interaction with TfR sensitive to the local environment (such as low pH in the endosomal compartment) would increase the chances of the biologic's dissociation from TfR—thus, in another way, reducing the size of the entity routed for exocytosis—although it can also decrease the probability of bivalent binding by reducing the chance that both arms of the mAb would bind TfR at the same time. All these considerations could be amended for other formats of biologics, but also for targets. For instance, would reduced affinity be relevant for brain-targeted peptides whose binding mode is intrinsically monovalent? Could crosslinking avoidance be less relevant for proteins with lower densities on the plasma membrane? While modeling can probably help with these or other similar questions, in the end, they can only be answered experimentally.

AUTHOR CONTRIBUTIONS

I confirm being the sole contributor of this work and have approved it for publication.

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