Review

Strategies for Transcytosis-Based Delivery Across the Blood-Brain Barrier

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Strategies for Transcytosis-Based Delivery Across the Blood-Brain Barrier

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Abstract

Transcytosis across brain capillary endothelial cell (BCEC) layer enables brain delivery of therapeutic macromolecules and nanocarriers. Interplay of their own properties and the properties of BCEC transport targets or intracellular trafficking machinery affects brain uptake. This interplay becomes even more nuanced for macromolecules targeted to several proteins on BCEC, as well as for engineered nanoparticles and viral vectors with complex biological interactions that may involve multiple proteins on the surface and within BCEC. Unraveling the effects of this interplay is paramount to further improvements in delivery. This paper proposes a framework where the delivery of systemically administered large therapeutics across the blood-brain barrier is predicated on three components: availability on the surface of BCEC, trafficking across BCEC, and availability in the bloodstream. It then highlights how interactions between large therapeutics and their BCEC surface targets, but also intracellular proteins, may be adjusted to minimize unproductive trafficking routes or entrapment, thereby maximizing transcytosis across BCEC. Finally, it suggests strategies to increase the availability of large therapeutics in the bloodstream and outlines how these individual adjustments can be balanced to improve overall transport across the blood-brain barrier.

Keywords: blood-brain barrier; drug delivery; transcytosis; trafficking; nanoparticles; viral vectors

1. Introduction

Transcytosis across the blood-brain barrier (BBB) is one method to deliver therapeutics into the brain after systemic administration. This method is especially relevant to large therapeutics and nanocarriers – collectively referred to below as constructs – since they are unlikely to cross brain capillary endothelial cells (BCEC) by diffusion, and, in contrast to small molecules, even benefit from the well-developed medicinal chemistry toolkit to undergo diffusion-enabling modifications. In transcytosis, the construct enters the endosomal network of BCEC and, after intracellular trafficking, is released from the abluminal membrane, deeper into brain parenchyma. This process has traditionally been divided into adsorptive-mediated and receptor-mediated transcytosis (AMT and RMT, respectively); carrier-mediated transport is sometimes included as well, but here transcytosis will refer to vesicular transport only [1–5]. In AMT, the construct's endocytic entry into BCEC would be mediated by its non-specific, charge-based interactions with the BCEC surface, while in RMT this entry is enabled by the construct's specific interactions with a receptor protein on BCEC. AMT is commonly exemplified by the transport of lectins or histones, and RMT – by that of transferrin (Tf), insulin, etc. Naturally, in such exemplifications, it is assumed that all these molecules are transcytosed across BCEC in the first place; in this paper, I do not comment on these assumptions, as they are irrelevant to its scope.

Here, I suggest a framework that emphasizes three components of trans-BCEC delivery efficiency: availability on the BCEC surface, transcytosis across BCEC, and availability in the bloodstream. This structure departs from the conventional division into AMT and RMT. In particular, it strives to reflect multiple roles that the same surface molecules may play in the construct's interactions with BCEC.

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Furthermore, it aims to account for the balance between the construct's interactions in the periphery and its binding to and trafficking across BCEC. This framework also serves as a basis for several strategies that, I would propose, might improve the construct's transcytosis at BCEC but also, partially, interfere with its intracellular fate in the periphery and thus indirectly increase the overall brain transport.

A focus on transcytosis across BCEC – more precisely, transport within the BCEC endosomal network that is largely dependent on the construct's interaction with BCEC surface molecules – excludes many other approaches explored for trans-BBB delivery. Among them is transient paracellular opening, whether local, e.g., in focused ultrasound-mediated BBB opening (FUS- BBBO), or global – most prominently but not only via osmotic disruption [6,7]. This approach does have its place, and its FUS-BBBO implementation is rapidly advancing [8,9]. Transient increase in non-specific vesicular transport, as a delivery approach, is not as established, at least because findings demonstrating an increase in this transport without concomitant paracellular leakage are more recent - see e.g., [10] for the role of pericyte-secreted vitronectin, or [11,12] for the role of major facilitator superfamily domain-containing protein 2a. Attempts to exploit this transport have been reported [13–15] but, just like non-specific paracellular opening, are outside the paper's scope. Here, I also do not consider membrane fusion proposed for liposome-mediated delivery [16–18] or direct translocation through the cell membrane suggested, as one of the possible entry mechanisms, for cell-penetrating peptides [19,20].

Finally, the status of BBB transport in disease warrants a separate mention, since for drug delivery into the brain it is far more relevant than that in health. Specific transport mechanisms vary but non-specific BBB transport, as a rule, tends to increase [21–23]. This increase, however, ranges from profound in the brain tumor core to comparatively modest in neurodegeneration. Furthermore, given the frequently heterogeneous BBB disruption in disease, one may wonder if it is not more rational to build a delivery strategy around the lowest common denominator – i.e., an intact BBB. This especially applies to primary brain cancer, where relatively unfettered access to the tumor core belies far greater challenges posed by the infiltrative border from which the tumor recurs after resection [24,25]. In short, even though trans-BBB delivery would ideally be tailored to each disease, in practice this customization may be difficult to implement explicitly; implicitly, of course, the success of any strategy, however generic, depends on disease specifics.

2. Availability on the BCEC Surface

Adherence and retention, which, I would propose, together govern the construct's availability on the BCEC surface, are not equivalent – a construct binding a rapidly internalizing target with a high affinity would ensure the former, but likely not the latter. Greater surface availability - where the construct can be attached to surface molecules, briefly persist in the adjacent liquid layer, or be in transition between these two states - has several consequences. First, the construct may eventually be taken up by fluid-phase endocytosis - i.e., as part of the extracellular fluid taken into BCEC when an endocytic vesicle forms on the BCEC surface. One reason this route is inefficient for many circulating proteins, e.g., albumin or fibrinogen, is their negative charge in the bloodstream [26,27]. That is, electrostatic repulsion from the strongly negatively charged BCEC glycocalyx and plasma membrane already forms a barrier against the fluid-phase entry of such proteins, aside from the fact that non-specific endocytosis itself - referring here to spontaneous, trigger- or receptor-independent mechanisms - is diminished in BCEC. However, this charge- related restriction does not need to apply to artificial constructs - indeed, it is the effects of albumin cationization that led to the introduction of AMT as a concept [28]. Second, a construct bound to one protein on BCEC may be taken up as a byproduct of another protein's endocytosis. If we set the diameter of an endosomal vesicle budding off the plasma membrane to ~100-150 nm on average [29], then its surface area is ~31,4x10^3 – ~70,7x10^3 nm^2, and all of this area must originate from the plasma membrane. Then, if the lateral width of a protein on the membrane is ~4-8 nm, this means that even with intracellular adaptor-mediated cargo selection and enrichment within the vesicle, the protein initiating endocytosis will carry with it hundreds to thousands of other nearby surface molecules, depending on packing density. This may partially account for the overall recycling, which, incidentally, links this discussion

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to the potential role of recycling in the internalization of constructs targeting diverse types of BCEC membrane proteins, including those that do not require endocytosis for their function [30]. Third, the construct may even specifically interact - to be endocytosed - with proteins other than those it is deliberately targeted to, although this seems more probable in larger carriers owing to their pleiotropic interactions driven by the capsid surface of viral vectors or the protein corona of nanoparticles (NPs), as well as viral vectors. Finally, in the case of intentional dual or multi-targeting, increased surface availability enabled by one target may increase the chance of interaction with the other(s).

Adopting this reasoning has some theoretical implications. First, AMT (more precisely, the 'adsorptive-mediated' part of it) becomes just one instance of surface availability-improving strategies. That is, there may be no fundamental difference between anchoring a construct to BCEC through cationization (which essentially is charge-based targeting the negatively charged glycocalyx and plasma membrane), targeting certain molecules on BCEC glycocalyx, targeting membrane lipids, targeting BCEC surface proteins that might not themselves predominantly drive the construct's endocytosis into BCEC, or even targeting proteins that will drive this endocytosis - in the latter case, one can distinguish between the anchoring and internalizing roles played by the same protein. Furthermore, this framework de-emphasizes RMT in the conventional sense - where a receptor mediates endo- and transcytosis of the construct across BCEC [1,31] - and, instead, aims to reflect multiple roles that surface molecules, not necessarily those intentionally targeted, play on the luminal surface of BCEC. Those roles, in addition to internalization, may include anchoring in general, but also, more narrowly, various rearrangements of proteins interacting with the construct on the BCEC surface (see below). Some of these roles may be defunct in mono-targeting to rapidly endocytosed receptors, such as transferrin receptor (TfR) or low-density lipoprotein receptor (LDLR), unless the construct blocks or decelerates their endocytosis. However, they do exist conceptually, even in these cases, and their contribution may increase substantially for other targets. The quantitative balance of these roles may define transport efficiency, especially for constructs interacting with multiple surface molecules on BCEC - whether through intentional dual- or multi-targeting, or latently, as one could expect for larger carriers such as NPs.

There are some practical consequences as well. The most important one is that the factors relevant to targets initiating the construct's endocytosis - RMT targets in the conventional sense - can be partially decoupled from those applied to 'anchoring' proteins. For instance, the latter do not need to internalize rapidly (in fact, at all, although they will anyway do so eventually, at least as part of the recycling process). It is sufficient for the construct to bind those proteins at low or moderate - but likely not high - affinity. Then they would serve as a depot that will increase the construct's local concentration - not only at the depot itself but also in the liquid layer adjacent to the membrane surface - to increase the chance of the construct's uptake through BCEC overall endocytic activity, such as it is, or through engagement with other intentionally targeted proteins. The reason this affinity should probably not be high is not only to ensure that the construct can get detached from its anchoring protein, but also to reduce interference with the protein's biological function, whatever that may be. Here, one would also observe that the size-dependent barrier formed by BCEC's uncommonly thick glycocalyx [32–34] would logically work both ways – just as it hinders the construct's access to BCEC plasma membrane from the bloodstream [32], it may hinder release from the plasma membrane to the bloodstream, meaning that even after dissociation from the anchoring protein, the construct will have more time to get re-attached to the membrane, with the duration of this window proportional to its size.

In addition, if a certain protein has low specificity to BCEC but otherwise favorable properties (e.g., high expression and internalization rate), then targeting more specific proteins in its proximity may increase the construct's uptake into BCEC, even if those other proteins do not have an equally high internalization rate. This proximity will likely not be permanent – because if those proteins were always found near faster-internalizing ones, their own internalization rates could be faster – but it may be induced by bispecific construct binding. For instance, if ligand binding causes a rapidly endocytosed protein to heterodimerize with another protein, more specific but with slower internalization rate, one could potentially exploit the internalization rate of the former and specificity

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of the latter simultaneously. BCEC specificity aside, examples of such heteromerization are abundant – e.g., that of serotonin receptors 5-HT_{1A} and 5-HT_7 [35], leucine-rich repeat- containing, G protein-coupled receptor 5 and LDL receptor-related protein 6 [36] or CC chemokine receptor 5 and C5a anaphylatoxin receptor [37], although for trans-BBB delivery one might want to leverage protein combinations with limited involvement in signaling to minimize downstream effects. Clearly, it would be better if this arrangement were reversed – i.e., if a construct binding a specific, albeit slowly internalized, protein would itself cause heterodimerization with another, more rapidly internalized protein (even if it had less to no specificity to BCEC), thus causing rapid endocytosis of the resulting complex, because then one could target the first protein alone; to my knowledge, however, such heterodimerization is not common, at least not where the difference in the endocytosis rates of two proteins is clear-cut.

Finally, with intentional dual and multi-targeting one may explore the balance between two different roles simultaneously played by both (or several) targets - driving the construct's endocytosis specifically and enabling it non-specifically, through increased availability on the BCEC surface. This investigation, applied quantitatively and to different classes of targets, will likely produce noteworthy results when more data is available, but one can make some observations even based on existing findings – e.g., with dual targeting to TfR and CD98 heavy chain (CD98hc) in [38]. By now it has been shown in several works that, unlike TfR, which promotes faster transcytosis and higher brain uptake at earlier time points (and, subsequently, faster degradation in the brain parenchyma - again, partly due to faster uptake by parenchymal cells expressing TfR), CD98hc-targeted monoclonal antibodies (mAbs) have a much slower brain transport, but also longer retention in the brain – that is, unless the construct is also targeted to some surface proteins on brain parenchyma cells [39–43]. In this context, it is then remarkable that in [38], dual-targeted mAbs with TfR affinity set to 100 nM and Cd98hc - varied at 170 or 550 nM, had a higher brain uptake than a TfR-targeted construct with the same affinity of 100 nM already at the earliest time point. From the perspective of transcytosis rates alone, this is counterintuitive – given equal concentrations, early on a construct targeted only to TfR could be reasonably expected to achieve higher parenchymal levels than a dual-targeted one, exactly because the latter's transcytosis may be dragged down by the far slower rates of CD98hc. However, if one considers not only the transcytosis rate but surface availability as well, the higher brain uptake of dual-targeted mAbs, even early on, may not be so surprising - after all, if there is more of the construct in the immediate vicinity of the luminal membrane, then more of it can be transcytosed, even if the transcytosis rate per se is slowed down by the contribution of CD98hc. This is why the reference to the time point here is crucial – later on, brain uptake readouts may be increasingly affected by the behavior of transcytosed mAbs beyond the blood-brain barrier - which is a different category altogether, even if some of the factors that impact it are shared with those affecting availability at and transcytosis from the luminal surface of BCEC. Fig. S3 in [38], albeit *in vitro* and in an unrelated cell model, strongly points in that direction by indicating that the retention of CD98hc-targeted mAbs on the cell surface observed earlier in [40] is preserved in dual-targeted mAbs as well. From here, an inference to greater availability of dual-targeted mAbs, explaining greater brain uptake already at the earliest time point, seems natural, although this interpretation can only be fully validated by techniques such as *in vivo* microdialysis [44,45] with measurement starting right after construct administration, and even then, a separate control to stain the construct on brain capillaries as a function of time would be warranted.

3.Trafficking Across BCEC

3.1. Trafficking with One vs Multiple BCEC Proteins

One speculative approach to enhance transcytosis could be construct redirection in which, briefly, a construct, after dissociation from its luminal surface target within the endosome, may bind another target, one more likely to deliver it from the endosomal network to the abluminal membrane [30]. Binding in this approach would possibly be environment-dependent, high on the cell surface and low in the endosomal milieu for the first target, and the other way around for the second one, to ensure that the second target, whether it is membrane-bound or secreted, would not intercept the construct in the periphery. This decoupling of endocytosis from transcytosis appears versatile in the

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sense that it allows diverse strategies to interfere with the construct's trafficking - obviously, at the expense of targeting at least two molecules. This targeting does not need to occur in the early or common recycling endosome only – while these locations, especially the latter, seem the most natural to redirect the construct, depending on the target's predominant trafficking route the construct might be made to dissociate from it in the trans-Golgi network (TGN) to bind proteins destined for secretion on the abluminal side. In addition, it does not need to be explicit - it may well be implicit and driven by the construct's intrinsic properties, as in the case of mAbs dissociating from their luminal surface target to bind neonatal fragment crystallizable (Fc) receptor (FcRn), adeno-associated viruses (AAVs) undergoing the same process to bind AAV receptor, etc. Furthermore, for larger constructs, in particular, one can also envision that this targeting may not even need to create new interactions – it may work by eliminating some of the construct's existing ones, those that impede its transcytosis. Nevertheless, this strategy still involves interaction with more than one molecule, or inversion thereof. Let us, however, entertain a narrower case where one and only one target - expressed on the luminal surface of BCEC - is meant to move the construct across the cell and release it into brain parenchyma.

First, one can consider constitutive transcytosis in the context described in [46,47] - where a protein with polarized localization is first inserted into one membrane and later transcytosed to its final destination on the opposite membrane. The importance of this mechanism is debatable even in BCEC-unrelated cells [48], and even if it does exist for some number of potential targets in BCEC, it might not be easily co-opted for construct transcytosis, since the mechanism itself implies that the targets will only appear transiently on the luminal surface and, in the end, will be located largely on the abluminal membrane – which may limit their use for trans-BCEC delivery. It may be more efficient to exploit the transcytosis of those targets that have substantial presence on the luminal surface of BCEC.

Consider a luminal surface target whose trafficking does not substantially deviate from the recycling pathway, e.g. by ubiquitination-driven lysosomal routing or moving further into the TGN TfR, in fact, is a good example. Let us postulate that this target's recycling may presuppose a certain probability of transcytosis - which, in a way, is recycling to the opposite surface. Let us also assume that the construct will not negatively interfere with the target's trafficking - e.g., via crosslinking through bi- or multivalent binding. One can then envision three scenarios for constructs endocytosed with such targets:

- dissociation within the endosome,
- recycling to the luminal membrane, and
- trafficking to the abluminal membrane

with the construct still bound to the target throughout its passage within BCEC in the latter two cases, at least intermittently. Dissociation within the endosome might not necessarily preclude transcytosis and, exactly as argued above, might even be exploited to improve it. Furthermore, depending on affinity and the local concentration, much of the construct may be expected to re- bind the target. For now, however, let us assume that this dissociation, especially a permanent one, will entail the construct's eventual degradation. If so, transport across BCEC becomes the balance of these three processes and, if one can eliminate the first, the share of the latter two - crucially, the last one, i.e., transcytosis, - will increase. In more specific terms, this means engineering the construct to allow dissociation on the abluminal membrane, but not in the endosomal milieu. Even more narrowly - within the currently available technical toolset - it implies low or moderate affinity at a neutral pH and high affinity at an endosomal pH. This goes contrary to the current trend in pH-dependent binding where constructs are engineered for higher affinity at neutral pH than in the endosome [39,49,50] as well as the notion that high-affinity binding causes entrapment within BCEC [51–53]. I would, however, argue that there is no contradiction, for two reasons. First, fundamentally, high affinity might only hinder construct transcytosis where it prevents dissociation on the abluminal membrane of BCEC (or, in pH terms, at a neutral pH). For instance, specifically in the case of the Tf-TfR complex, high-affinity binding of apo-Tf to TfR does not impede TfR's return to the plasma membrane, where apo-Tf, losing its affinity to TfR, is released. Second, in more practical terms, high affinity in the endosome might further compound the issue if the construct itself induces the target's lysosomal

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routing, whether by forming exceedingly large complexes or for any other reason. In those cases, dissociation within the endosomal network would indeed be a benefit; however, the strategy considered here specifically excludes this negative interference as a precondition (see above). Curiously – and only for argument's sake - if one does subscribe to the view of Tf transcytosis across the BBB being the quantitatively substantial or dominant outcome of Tf-TfR complex trafficking in BCEC [4,54] and extends this view to TfR's interaction with artificial constructs as well, this approach might seem even more beneficial, combining easier dissociation on the abluminal membrane with the lack of - presumably unproductive - dissociation in the endosome.

Clearly, this hypothetical approach, if at all successful, would hinge on the specifics of the target's – although not necessarily its endogenous ligands' - constitutive intracellular trafficking route. Unlike the construct redirection strategy, which aims to bypass this factor, here it becomes paramount, and one can foresee that it may fail with targets that have a substantial contribution of the degradative pathway. Furthermore, it may not increase transcytosis specifically, but rather in an inextricable link with recycling. In the periphery, this might well be an advantage, as I argue in the next section. In BCEC, however, recycling to the luminal surface is unproductive - perhaps not as unproductive as lysosomal degradation, but still suboptimal at best. Indeed, in cases where the construct would rely on one target only for its transcytosis, i.e., would not leverage other proteins in the endosomal network, I envision that when most of the avoidable hindrances - crosslinking, lack of dissociation on the abluminal membrane, meandering or simply dead-end trafficking routes, etc. – are addressed either by construct optimization or by choosing other targets, it is recycling to and release from the luminal membrane that will become the final bottleneck capping construct transcytosis at BCEC. This is because of all cellular processes it is the one most closely resembling transcytosis in nearly every aspect but the direction - and thus, perhaps, the most difficult to isolate and eliminate. One hypothetical exception could be conformational changes deliberately induced by interaction with artificial constructs, e.g., masking the target's apical sorting motifs to avoid recognition by intracellular trafficking machinery and thus redirect the construct toward the abluminal membrane. On top of that, making those changes specific to BCEC (i.e., avoiding similar behavior in the periphery - see below) would, I expect, dramatically increase brain transport; however, this would require a level of specificity that the intracellular trafficking machinery of BCEC may or may not be able to offer.

3.2. Trafficking of Large Constructs

If we postulate that beyond a certain size limit, carriers such as NPs and some viral vectors may be simply too large for transcytosis through the endosomal network [30], especially in its tubular part, then what routes remain? Lysosomal entrapment is typically not desired, and endosomal escape of NPs within BCEC is premature - except, possibly, for making BCEC synthesize therapeutic proteins [55–57] or, more speculatively, for the subsequent transport of small molecules comprising the payload from cytosol across the abluminal membrane. One possible approach might be endosomal release – where, briefly, NPs could be engineered for releasing their payload in the endosome without triggering endosomal escape, so that the payload, rather than NPs, would continue trafficking through the endosomal network. However, this hypothetical approach may only deliver NP payload, not intact NPs across the BBB. For intact carriers above a certain size limit, the only remaining option seems to be exploiting those end-to-end paths across the entire endosomal network that are limited to vesicular transport. Furthermore, at no point along the path should the vesicle size drop to levels that can no longer accommodate the construct – this is important because the diameter of some types of intracellular vesicles, e.g., those involved in retrograde transport, is equal to or only marginally greater than that of the tubular network [58]. There are several possibilities, but the most notable one is exocytosis of multivesicular bodies (MVBs). Let us consider it.

First, recall that topologically, the outer leaflet of the intraluminal vesicle (ILV) membrane within an MVB is equivalent to the inner leaflet of MVB limiting membrane, and the content of those vesicles - to the cytosol. If we ignore relatively far-fetched cases, such as membrane translocation, this means that NPs transitioning to MVBs from early endosomes cannot be within ILVs but can be attached to the ILV outer leaflet. In the end, however, MVB exocytosis releases its entire content, i.e., that includes

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not only ILVs (which at that point become extracellular vesicles, or EVs), but also, possibly, NPs present in the lumen of the MVB. The question is then how exactly MVBs are formed in BCEC, and, in more practical terms, whether endocytosed NPs can be preferentially transported to MVBs that are destined for exocytosis. This, in turn, hinges on the cardinality of the relationship between early endosomes and MVBs.

For a one-to-one relationship, where one early endosome evolves into one MVB – the classic maturation model [59] - the fate of NPs contained within such early endosomes appears largely predetermined, except, perhaps, for retrograde transport to the TGN, assuming that the vesicles involved in this transport can accommodate the NPs. This predetermination does not imply that NPs will necessarily be trapped within the cell (although that would be highly probable) - simply that there is not much to do here in terms of path bifurcation, at least at this stage of trafficking. However, for cases where one early endosome may produce several MVBs – as in the stable compartment model [59–61] - it is interesting whether or how NPs could be directed to the desired type. Recall here that in such scenarios, a non-tubular region of early endosomes would curve outward and undergo fission to form (prospective) MVBs. If the fate of MVBs can be either degradative or exocytic, then the logical strategy will be to target the inner leaflets of those regions of the early endosome limiting membrane that are more likely to give rise to MVBs whose content will eventually be exocytosed. What would those regions contain? EVs themselves may provide the answer - since their membrane is formed by invagination of the limiting membrane of MVBs, the transmembrane proteins enriched in the EV membrane are largely inherited from the limiting membrane of parent MVBs and, by extension, from 'grandparent' early endosomes. Here, the obvious targeting choice would be endosomal vesicular domains of some canonical EV transmembrane proteins – e.g., tetraspanins [62]. Evidently, these EV proteins must have a vesicular sequence in the first place, because otherwise there will be nothing for NPs to bind from within the endosomal lumen – e.g., unlike tetraspanins, some other common EV markers such as Alix would be of no use in this context. Furthermore, this choice might potentially be even better if recruitment of suitable EV marker proteins into the membrane of ILVs did not involve their selective enrichment on the limiting membrane of MVBs, because then it would make interaction on the limiting membrane of early endosomes even more specific. Even with selective enrichment, however, they or other proteins with domains present on the outer leaflet of the ILV membrane (only the outer leaflet is strictly relevant here, but in practical terms this largely means transmembrane proteins of EVs) would be a natural target to anchor the NPs to on the limiting membrane of early endosomes.

3.3. Trafficking Interference Points

Let us summarize the strategies described above and group them to reflect the stages in trafficking where construct optimization, another target, or both may reduce unproductive trafficking routes and intracellular degradation and direct the construct toward the abluminal membrane of BCEC with release into brain parenchyma (Table 1). The strategies in Table 1 can be broadly divided based on whether they simply adjust or introduce interactions with surface or intracellular targets, or deliberately modify the behavior of those targets - e.g., in how these targets themselves interact with other BCEC proteins. The former, in a way, exploit BCEC targets as moving platforms throughout the construct's journey across BCEC, to bind to and dissociate from at appropriate milestones, all the while avoiding interference with the targets' natural trafficking. The latter requires designing precise conformational changes, which makes it far more difficult than generating constructs capable of binding a given protein, even with epitope binning. This challenge is further augmented if the changes need to be introduced in the endosomal network, since it would require adjustment for an acidic pH (see below). For binding on the cell surface, however, this was implemented in [63] - to mimic conformational changes induced by a receptor's endogenous ligand. More speculatively, it might be employed to modify interactions with intracellular adaptors triggering endocytosis - for luminal surface targets with slow internalization rates that may, in their cytoplasmic tails, have hidden endocytic motifs that conformational changes caused by the construct's binding to an extracellular epitope may help expose.

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The approaches below do not include the ones already demonstrated for transcytosis improvement - such as low or moderate affinity binding that aims to facilitate release from the abluminal membrane [51,52], or the monovalent binding mode [64] that aims to prevent large complex formation and preserve the target's native intracellular trafficking. They also exclude various explorations of these themes, especially those in large constructs. For nanocarriers, in particular, the strength of the construct's interaction with BCEC targets is more likely to be modulated through ligand density (i.e., in effect, avidity) [65,66] rather than only the affinity of targeting moieties. In itself, however, it is similar to affinity modification in smaller constructs, except that NPs are far less likely to achieve the purely monovalent binding mode that is arguably more compatible with the trafficking of recycling receptors, nor can they easily avoid size-dictated trafficking constraints – although their large loading capacity allowing the release of many payload molecules for a single transcytosis event can to some extent make up for these impediments. A more interesting avenue could involve varying the geometric pattern of ligand distribution on the construct surface and investigating its effects [67]; at this stage, however, commenting on this, as well as on many other types of modifications [68] whose diversity (although not combinatorial complexity) in synthetic NPs is far greater than that in protein-based constructs could be premature in the context of brain delivery.

Table 1. Strategies for construct transcytosis across BCEC.

Entity	Location	Description	Rationale
Luminal surface target	PM, EE, RE, less likely MVB	High- or moderate affinity binding at cell surface, low in the endosome	Dissociation in the endosome, possibly for subsequent interaction with endosomal proteins
Abluminal surface proteins	EE, more likely RE	High affinity in the endosome, low on the cell surface	Capturing the dissociated construct in the endosome and redirecting it toward the abluminal membrane for release into parenchyma
Endocytic machinery proteins, vesicular domain*	EE or RE	Moderate affinity in the endosome	As above; the difference is that for intracellular machinery, peripheral expression-dictated specificity constraints may be less relevant.
Abluminal surface proteins or secreted proteins	TGN	High affinity in the TGN, low on the cell surface**	Capturing dissociated construct in the TGN and redirecting to the abluminal membrane or for secretion into brain parenchyma
Transmembrane proteins of EVs	EE	High affinity in the endosome, low on the cell surface***	Tethering to those EE regions that are more likely to give rise to exocytosis-destined MVBs
Luminal surface target	EE, RE	Conformational change to mask apical sorting signals	Preventing recycling to the luminal membrane, thus indirectly promoting transcytosis
Luminal surface target	PM	Conformational change to trigger endocytosis, e.g., by exposing possibly inactive endocytic motifs	Inducing endocytosis (for proteins with slow internalization rate but otherwise favorable properties)

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Luminal surface target	PM, EE, RE	Moderate affinity on the cell surface, high in the endosome	Eliminating intracellular dissociation - for targets with a strong recycling functionality - while preserving release on the cell surface
Construct (nanocarriers)	EE, or as early as possible	Triggering endosomal release of the payload	Released payload may traverse endosomal structures that are too narrow for the construct. The payload itself may be engineered to interact with endosomal proteins, as above.

* - naturally, only those proteins of the trafficking machinery that have sizable or at least non-degenerate vesicular tails ** - Ideally, binding to the luminal surface target would be dependent on something other than pH only - e.g., certain ionic concentrations - to avoid dissociation long before reaching the TGN. Also, for secreted proteins, low affinity on the BCEC surface may be irrelevant per se, but still useful for construct dissociation in the parenchyma. *** - low affinity on the cell surface may be irrelevant to MVB exocytosis but may prevent the construct's unwanted interactions with the target on the plasma membrane, especially since canonical EV proteins have low specificity. Abbreviations: PM – plasma membrane, EE – early endosome, RE - recycling endosome, TGN – trans-Golgi network.

There are several observations to be made based on this list. The first one is topological. The strategies above, for the most part, view transcytosis across BCEC as a passage within a fully enclosed space, which serves as an approximation of the tubulovesicular network spanning endosomes and the TGN. This network, especially in its endosomal part, may well be the most efficient transcellular transport route and may yet have much to offer in terms of construct optimization toward greater transcytosis efficiency. It has, however, only a subset of mechanisms that may potentially be used to enhance transcytosis. Others may involve, e.g., the construct's return to the endosomal network after its endosomal escape into the cytosol (for instance, by moving back to an MVB or, more precisely, an ILV), or even unconventional secretion from the cytosol [69], e.g., through ectosomes, although the quantitative importance of these mechanisms is rather uncertain and it might be questionable what improvements, if any, they can bring.

In addition, I do not consider strategies that permanently or even transiently alter BCEC's endogenous trafficking machinery. This includes overexpression, silencing or any other approaches whose biological effects may go far beyond interaction with therapeutic constructs. The obvious reason is that they may pose a major risk outside the BBB. However, even if the effect is limited to BCEC, the consequences may still be unpredictable – and in that case one might also wonder why the same targets that deliver those interference agents (typically nucleic acid-based) into BCEC cannot be used to deliver therapeutic constructs across, which would likely be safer.

Furthermore, some of the strategies in Table 1 call for environment-dependent binding outright, while for others it may be optional but still advantageous. It would seem that engineering constructs for high affinity binding in the endosomal milieu and low or moderate on the cell surface – not necessarily to the same target - may open numerous possibilities for manipulating the construct's intracellular trafficking without interfering with interactions on the cell surface. Sadly, most of the new-generation protein design tools are currently of limited use here because the bulk of the data they were trained on reflects binding at a neutral pH. Developing new tools or building new training datasets may greatly advance strategies that hijack intracellular processes - and, in addition, reduce the risk of negatively affecting the construct's interactions in the periphery.

Finally, one will observe that many considerations above are applicable to transport across other blood-tissue barriers. If anything, although from a practical perspective, the BBB is a far more formidable obstacle in brain delivery than other vascular barriers - in delivery to their respective tissues, theoretically it appears to be a much simpler case - precisely because of the nearly perfect exclusion of non-specific large molecule transport, transcellular or paracellular, which reduces the number of processes one has to consider, quantitatively as well.

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4. Availability in the Bloodstream

The construct's availability in the bloodstream is primarily affected by its pK profile in the periphery, although processes that return an intact construct from brain parenchyma back to the bloodstream, as well as the construct's adherence and retention on the BCEC surface may also have an impact. In this paper, I will only touch upon the cellular component of the construct's peripheral pK – especially its target-mediated clearance in unrelated tissues, as one of the manifestations of target-mediated drug disposition. The notion is simple - the same target that is used for the construct's transcytosis at BCEC may mediate its binding and uptake by peripheral cells expressing the target [70,71]. Brain parenchyma targets may do the same, unless they are completely specific to the brain or the moieties that are to interact with them are shielded within the construct, but for simplicity let us exclude that from consideration. It may seem logical to minimize such binding and uptake, and greater specificity of BCEC delivery targets can certainly be an advantage here, although, more broadly, this advantage is not absolute, since even without peripheral target engagement, the construct will still be delivered to unrelated tissues - or cleared - through non-specific physiological mechanisms [72]. Insofar as the problem exists, however, one may want to address it.

First, using mAbs as an example, for targets that have a strong expression both on BCEC and on cells expressing FcRn, pH-dependent binding to the BCEC target with dissociation at an endosomal pH, possibly with higher binding to FcRn at the same endosomal pH, might rescue the construct from degradation in the periphery. Ideally, this should not happen because it is vascular endothelial cells (as well as some myeloid lineage cells) that have the highest expression of FcRn [73], and those are exactly the cells where one would not want the BCEC target to be expressed, since they would be the ones most accessible to the construct after systemic administration. Ideals, however, are unattainable by definition, so for certain targets one might as well consider this dissociation-based approach. The rationale needs no further description, since it is basically equivalent to the recycling antibody concept originally proposed in [74] and implemented with modifications in [75,76], and the only semantic difference is that the delivery target in this case would play the role of an antigen in the original concept. The point, evidently, is to rid the construct of the hindrances that its binding to the delivery target creates for interaction with FcRn; in cells that do not express the delivery target, the construct will likely be taken up non-specifically in fluid phase, meaning that no intracellular hindrances should exist to begin with, and FcRn-mediated recycling - at whatever capacity various peripheral cells may enable it - will take over just as it would for untargeted mAbs, provided that the targeting moiety does not itself interfere with this recycling.

Second, as in the previous section, one can speculate that for some types of BCEC delivery targets - and constructs that do not interfere with their constitutive trafficking - higher affinity in the endosomal milieu coupled with low or moderate affinity on the cell surface might be a benefit. This may seem counterintuitive given that target-mediated binding and uptake by peripheral cells would typically be avoided. To the extent that any interactions in the periphery impede the construct's free circulation, that is reasonable. As far as target-mediated uptake is concerned, however, one would observe that it is not uptake per se that eliminates constructs from circulation, but rather irreversible intracellular entrapment and lysosomal degradation. That is, target-mediated peripheral uptake might not be completely detrimental if the same target returns and releases the construct from the cell surface. The key challenge with this approach in the periphery would lie in release from the plasma membrane – since the same molecule would internalize the construct and return it to the cell surface, the distance between the construct and its delivery target once the construct is back on the plasma membrane would be zero, so it is only the construct's low or moderate affinity at a neutral pH that would allow for some degree of release from the cell surface. This is in contrast to the recycling antibody concept where the return would be enabled by FcRn after dissociation in the endosome, and while the delivery target may well recapture the construct on the plasma membrane in that case too, there will still be some random distance between its location and the location of the construct that reappears on the membrane, and this distance will make the recapture less likely.

Direction-wise, the construct will optimally be released back into circulation; however, transcytosis to tissue parenchyma may not permanently block the construct from returning to the bloodstream either. Here one would also observe that transcytosis from the bloodstream across

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discontinuous endothelium may in any case be dwarfed by paracellular transport [77], rendering this factor moot, and, more broadly, that the distinction between recycling and transcytosis is only relevant to polarized cells and is clearly inapplicable to e.g., circulating blood cells. Even transcytosis to the epithelial lumen is, to some extent, reversible - megalin-mediated reabsorption from kidney proximal tubules being the prime example, even if for endogenous ligands this reabsorption is only followed by transcytosis to a minor extent and / or in certain cases. In short, anything that prevents permanent intracellular entrapment of circulating constructs may be beneficial from the pK perspective.

Nevertheless, recycling to the bloodstream is still the ideal scenario in the periphery. FcRn-mediated recycling, albeit the best-known case, especially in the therapeutic mAb field, is only an instance of this process. For other instances, one need not go further than TfR, and it is hardly a coincidence that Tf is among the longest-lived proteins in blood, with a half-life of approximately 8-10 days [78]. One would expect that this half-life could be even longer were TfR expressed on endothelial cells lining peripheral blood vessels, but a lack of this expression is exactly what forms the - still limited - specificity of TfR to BCEC. At a more abstract level, one may realize that these proteins merely act as agents of the recycling process itself and recall that morphologically this process is largely implemented through scission of vesicles from the tubular extensions of early or recycling endosomes, with their subsequent fusion with the plasma membrane. Recruiting a construct into those intracellular structures may then increase the chance of their return to the cell surface. This may open possibilities for interference that go beyond FcRn, TfR, Ldlr and other members of its family, asialoglycoprotein receptor, luminal domains of (other) endosomal proteins or, indeed, proteins in general, toward more universal targets present in every cell. For instance, even though the specificity of lipids to different intracellular structures might not be as high or as easily exploited as that of proteins, tubular extensions are enriched in phosphatidylinositol 4- phosphate [79–81] (through conversion from endosomal phosphatidylinositol 3-phosphate), which, one could speculate, might be used as a target in this context, especially if bound at an endosomal pH with dissociation at a neutral pH – to avoid binding at the plasma membrane where it is present as well. Going beyond molecular entities, even the very curvature of those extensions (clearly higher in the tubular than in the vesicular compartment because curvature is inversely proportional to the radius) may become a target if the constructs can be engineered to sense it - which, given recent advances in research into curvature sensing and binding [82,83], may not be nearly as futuristic as one might think.

5. Conclusions

As transcytosis-based approaches to delivery across the BBB continue to evolve, construct optimization through modification of properties such as affinity, valency, pH-depending binding - or, in the case of larger carriers, ligand density, size, or even protein corona - leads to deeper mechanistic insights into the effects of those properties on the construct's interactions with BCEC. In addition, new delivery targets or combinations of targets are identified and tested experimentally, and findings elucidating the different transport profiles enabled by these targets enhance our understanding of BCEC trafficking. Finally, more sophisticated characterization methods allow probing the construct's transcytosis across BCEC with increasing granularity and at all levels - from visualization of the construct's adherence and trafficking to comprehensive characterization of interactions between biologically complex constructs and various BCEC molecules. These developments may call for new ways to view transcytosis at BCEC and seamlessly reflect the different roles that various BCEC molecules - sometimes the same ones - might play in the construct's journey across BCEC, from attachment to the glycocalyx or the luminal membrane through endocytosis and intracellular trafficking to release into brain parenchyma. The construct's cellular interactions - and the interplay of these interactions - at BCEC should also be viewed in a balance with those outside it, especially in the periphery, since it is this balance that defines the construct's overall brain uptake after systemic administration. Above all, any framework to view the construct's transcytosis should aim not only to explain new experimental observations, but also to formulate more refined delivery approaches that incorporate new insights that these observations help generate. In this paper, I sought to propose one such framework and use it to suggest several strategies for improved transport across the BBB.

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