Design, expression, and analysis of antibody-based blood-brain barrier shuttles

ANDRÉS DE LA ROSA
Antibody therapeutics, with their strong and highly selective target binding, are now used to treat various diseases. However, to enable their use to treat brain disorders, they must be delivered across the blood-brain barrier (BBB), as without active transport, only around 0.01% of intravenously injected doses reach the brain. Brain delivery can be done by BBB shuttles capable of binding receptors that naturally transport proteins, e.g., the Transferrin receptor (TfR). This thesis has studied strategies for designing TfR-binding shuttles and how to enhance the protein expression of antibody therapeutics. In Paper I, we shared our updated transient gene expression (TGE) protocol and developed a small-scale version to surmount the cost limitations of testing many conditions. Large variations of protein expression were observed for both protocols, prompting future studies investigating its cause(s). In paper II, we investigated if binding to the glycosaminoglycan heparan sulfate (HS) present at the BBB could improve brain delivery. Our results indicate that the BBB shuttle scFv8D3 is not dependent on the HS-binding sites identified, and adding new HS-binding sites did not enhance delivery. However, further studies are required due to HS's complexity and heterogeneity. Decreasing the TfR affinity of BBB shuttles has been shown to boost the delivery of therapeutic doses of high affinity anti-TfR antibodies, e.g., bivalent 8D3 antibodies. In Paper III, we applied the strategy to a monovalent single-chain fragment variable (scFv) of 8D3 (scFv8D3) based BBB shuttle. Our affinity mutants exhibited lowered TfR affinity, longer blood half-life, and higher brain concentration. Using our In-Cell BBB Trans assay, we concluded that the increased brain concentration is likely due to extended blood half-life. In paper IV, we fused the TfR ligand holo-transferrin to the TfR binding arms of the partly bivalent RmAb158-scFv8D3 antibody. Our results indicate that the TfR binding shifted from partly to fully bivalent, resulting in markedly decreased in vitro transcytosis. The potential transcytosis-promoting effect of the fused holoTf was absent and/or counteracted by the bivalent binding of the design. However, the strategy may still prove useful for monovalent TfR binders.

In conclusion, monovalent and low-to-moderate affinity are likely beneficial binding properties for TfR-mediated brain delivery at therapeutic doses. However, whether it is possible to enhance brain delivery with HS-binding or holoTf-fusion requires further study.

Keywords: Antibody therapeutics, transient gene expression, protein production, blood-brain barrier (BBB), BBB shuttle, brain delivery, Transferrin receptor (TfR), heparan sulfate, holo-transferrin

Andrés de la Rosa, Department of Pharmacy, Box 580, Uppsala University, SE-75123 Uppsala, Sweden.

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This thesis is based on the following papers, referred to in the text by their Roman numerals.


III. de la Rosa, A., Metzendorf, N., Efverström, J., Morrison, J., Hultqvist, G. (2023) Lowering the affinity of single-chain monovalent BBB shuttle scFc-scFv8D3 prolongs its half-life and increases brain concentration. Manuscript for submission

IV. de la Rosa A, Morrison J, Hultqvist G. (2023) Fusion of holo-transferrin to the TfR-binding BBB-penetrating antibody therapeutic RmAb158-scFv8D3 decreases its in vitro transcytosis. Manuscript for submission

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## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Antibody therapeutics for the treatment of brain disorders</td>
<td>11</td>
</tr>
<tr>
<td>Proteins</td>
<td>11</td>
</tr>
<tr>
<td>Expression of recombinant proteins</td>
<td>13</td>
</tr>
<tr>
<td>Transient gene expression (TGE)</td>
<td>14</td>
</tr>
<tr>
<td>Antibody structure</td>
<td>15</td>
</tr>
<tr>
<td>Antibody engineering and binding properties</td>
<td>16</td>
</tr>
<tr>
<td>The challenge of crossing the blood-brain barrier (BBB)</td>
<td>18</td>
</tr>
<tr>
<td>Heparan sulfate (HS) and HS proteoglycans (HSPGs)</td>
<td>19</td>
</tr>
<tr>
<td>Transport through the blood-brain barrier (BBB)</td>
<td>21</td>
</tr>
<tr>
<td>Receptor targets for the molecular Trojan horse strategy</td>
<td>22</td>
</tr>
<tr>
<td>Transcytosis of transferrin (Tf) via transferrin receptor 1(TfR)</td>
<td>23</td>
</tr>
<tr>
<td>Properties of TfR-binders influencing brain delivery</td>
<td>27</td>
</tr>
<tr>
<td>Reducing TfR affinity of monovalent TfR binders to improve brain delivery</td>
<td>30</td>
</tr>
<tr>
<td>Monovalent TfR antibody design</td>
<td>30</td>
</tr>
<tr>
<td>The monovalent BBB shuttle scFv8D3</td>
<td>31</td>
</tr>
<tr>
<td>Methods</td>
<td>33</td>
</tr>
<tr>
<td>Protein design</td>
<td>33</td>
</tr>
<tr>
<td>Protein expression and purification</td>
<td>34</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
<td>35</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>35</td>
</tr>
<tr>
<td>Quantification ELISA</td>
<td>35</td>
</tr>
<tr>
<td>Transferrin receptor (TfR) ELISA</td>
<td>36</td>
</tr>
<tr>
<td>Thermal shift assay</td>
<td>36</td>
</tr>
<tr>
<td>Heparin column liquid chromatography</td>
<td>36</td>
</tr>
<tr>
<td>In vitro BBB transcytosis assay</td>
<td>37</td>
</tr>
<tr>
<td>Radiochemistry</td>
<td>37</td>
</tr>
<tr>
<td>In vivo experiments</td>
<td>37</td>
</tr>
<tr>
<td>Capillary depletion</td>
<td>38</td>
</tr>
<tr>
<td>Nuclear track emulsion (NTE) autoradiography</td>
<td>39</td>
</tr>
<tr>
<td>Thin-layer chromatography (TLC)</td>
<td>39</td>
</tr>
<tr>
<td>Aims</td>
<td>40</td>
</tr>
</tbody>
</table>
Abbreviations

\( ^{125}\text{I} \) Iodine-125
N-terminus Amine group
BBB Blood-brain barrier
AMT Adsorptive-mediated transcytosis
BBB Blood brain barrier
BEC Blood endothelial cell
C-terminus Carboxyl group
CH Constant heavy domain
CL Constant light domain
CDR Complimentary Determining Regions
CSF Cerebrospinal fluid
DMT-1 Divalent metal transporter 1
ELISA Enzyme-linked immunosorbent assay
EC Endothelial cell
E.coli *Escherichia coli*
ECM Extracellular matrix
Fab Fragment antigen-binding
Fc Fragment crystallizable
Fe\(^{3+}\) Ferric iron
Fe\(^{2+}\) Ferrous iron
G4S Glycine-serine linker
HRP Horse-radish peroxidase
HS Heparan sulfate
HSPG Heparan sulfate proteoglycan
Ig Immunoglobulin
ILV Intraluminal vesicle
KD Dissociation constant
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PC</td>
<td>Pericyte</td>
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<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>PTM</td>
<td>Post-translational modification</td>
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<td>R-group</td>
<td>Functional group</td>
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<td>RME</td>
<td>Receptor-mediated endocytosis</td>
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<td>RMT</td>
<td>Receptor-mediated transcytosis</td>
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<tr>
<td>scFc</td>
<td>Single chain fragment constant</td>
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<tr>
<td>scFv</td>
<td>Single chain fragment variable</td>
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<td>SDS</td>
<td>Sodium dodecylsulfate</td>
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<td>TGE</td>
<td>Transient gene expression</td>
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<td>Tf</td>
<td>Transferrin</td>
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<td>TfR</td>
<td>Transferrin receptor</td>
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<td>TV</td>
<td>Transport Vehicle</td>
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<td>VH</td>
<td>Variable heavy chain</td>
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<tr>
<td>VL</td>
<td>Variable light chain</td>
</tr>
<tr>
<td>VHCDR</td>
<td>Variable heavy chain CDR</td>
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<tr>
<td>VLCDR</td>
<td>Variable light chain CDR</td>
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</tbody>
</table>
Introduction

Antibody therapeutics for the treatment of brain disorders

Antibodies, also known as immunoglobulins, are a crucial component of our immune system and help to protect us from pathogens such as viruses and bacteria that enter the body\(^1\). Antibody therapeutics refer to protein molecules that contain at least one binding site obtained from an antibody gene. These molecules are typically larger and more complex than small-molecule drugs, offering highly specific target binding with strong affinity. Due to their unique binding specificity and fewer off-target effects, they generally cause fewer side effects compared to other drugs\(^2\). The field of antibody therapeutics has seen tremendous growth due to its remarkable potential in treating various diseases. Currently, there are almost 1200 antibody therapeutics in clinical trials, out of which 175 are either approved or under regulatory review\(^3\). However, out of these, only a handful target brain diseases due to the challenge of crossing the blood-brain barrier (BBB), which hinders large molecules from reaching the brain from the blood circulation\(^4,5\). Therefore, developing antibody-based blood-brain barrier shuttles capable of delivering antibody therapeutics is paramount for enabling the treatment of brain disorders. The main focus of this thesis was to investigate important binding properties of BBB shuttles and strategies for improving their performance (Paper II, III, and IV), with a secondary focus on improving their expression in cell-based production (Paper I). Producing sufficient protein for experimental work can often be a limitation when developing antibody therapeutics.

Proteins

Proteins are the executors of the genetic information encoded by DNA; they are polymers built up by 20 different amino acids, and being the most diverse of all macromolecules, they can carry out the necessary tasks needed for life\(^6\). In the 19th century, chemists recognized the importance of proteins, and the term protein, coined by the chemist Jöns Jacob Berzelius in 1838, is derived from the Greek word próteios, which means "of the highest rank"\(^7\). The building blocks of proteins, namely the aforementioned amino acids, all share a
common structure consisting of a central carbon which is bonded to a carboxylic group (C-terminus), an amine group (N-terminus), hydrogen, and a unique side chain specific for each of the 20 amino acids (Figure 1A). Amino acid side chains, also referred to as functional groups (R-groups), vary greatly, and the R-groups determine the characteristics of the amino acid in terms of chemical, structural, and functional properties. R-groups are categorized by properties such as net charge, polarity, hydrophobicity, and whether they have an aromatic structure. During protein synthesis, amino acids become covalently linked to other amino acids by peptide bonds between C- and N-termini, forming a polypeptide chain (Figure 1A). The sequence of amino acids within a polypeptide chain is referred to as its primary structure (Figure 1B), and this primary structure determines how the polypeptide chains fold into secondary (Figure 1C) and tertiary higher-order structures (Figure 1D). Hydrogen bonds and Van der Waal interactions between the peptide backbone of the polypeptide chain fold the protein into secondary structures, the most common ones being α-helices and β-sheets (Figure 1C). The secondary structures are often connected by unstructured stretches of hydrophilic amino acids forming flexible loops. Multiple secondary structures may interact to form tertiary structures by forming hydrogen bonds, hydrophobic/hydrophilic interactions, ionic bonds, and disulfide bridges. These interactions fold the protein to reach a stable low free energy state. Larger proteins containing multiple domains often require the assistance of molecular chaperones, which are proteins that can assist in various forms, including stabilizing folding intermediates during or after the protein synthesis. A fitting example of large proteins is antibodies, which are comprised of four peptide chains in which multiple domains need to be correctly folded.
Expression of recombinant proteins

In 1973, Cohen and colleagues discovered DNA cloning, allowing for recombinant protein production. The discovery made it possible to clone a genetically manipulated gene into vectors like plasmids and express it in hosts different from the original. This enabled not only the expression of natural proteins but also the design of protein constructs by editing the DNA sequence of the gene of interest. The hosts used as expression systems include prokaryotic organisms such as bacteria and eukaryotic organisms such as yeast, plant, and mammalian cells into which a plasmid containing the gene of interest is introduced. The plasmid is designed in such a way as to increase protein expression by using strong promoter sequences such as the cytomegalovirus-promoter. Escherichia coli (E. coli) is the most popular bacterial expression system due to its high protein production capacity, fast growth, and ease of culture, making it the most cost-effective expression system. However, for producing antibody therapeutics that require the correct folding of multiple domains and
post-translational modifications (PTMs), E. coli is not preferred as it lacks the necessary cellular machinery to meet these requirements. Additionally, E. coli have limited protein secretion, which can cause the accumulation of expressed protein within the cytoplasm, often resulting in protein aggregation into inclusion bodies, severely decreasing the protein yields. Instead, mammalian cells are commonly used to produce antibody therapeutics as they can provide native folding and PTMs while also being efficient at secreting produced proteins into the culture medium. The drawback of mammalian expression systems is their slow growth, demanding culture requirements, and expensive growth media and culture media, making them significantly less cost-effective than bacterial expression. Stable mammalian cell lines are used when producing large amounts of antibody therapeutics, as they can produce up to several grams of protein per liter of transfection. Stable cell lines are established through a clonal selection process in which the gene of interest is incorporated into their genome. However, this process generally takes several weeks up to months and is therefore not commonly used during research phases. To facilitate the development of new drug candidates during research phases, it is advantageous to use transient gene expression (TGE).

Transient gene expression (TGE)

TGE can produce up to milligrams or even a few grams of proteins per liter of transfection within 4-12 days. TGE works by transfecting host cells with a vector (or multiple vectors) containing the gene(s) one wishes to express. This technology relies on the vector, often a plasmid, being delivered into the host cells' nuclei, resulting in transient gene expression since the vector is not duplicated when the cell divides. Several methods have been explored to deliver plasmid DNA into host cells, including chemical, viral, and physical delivery methods. However, all methods confer some measure of toxicity, which may affect protein expression. Though TGE has been shown by our research group and others to produce workable quantities of many proteins, systems can always be improved to increase protein production output, which could save costs by decreasing the size of transfections needed and help achieve sufficient protein yields for experimental work.

In part I of Paper I, we detail and share improvements to our previously published and highly cited protocol for TGE of antibody therapeutics. In part II of Paper I, we developed a small-scale version of the TGE protocol to overcome the cost limitation of testing many cell culture conditions. To test the small-scale protocol, we studied the effects of adding potential protein enhancers to improve protein expression. The substances we tested belong to a class of molecules known as longevity molecules, which are believed to extend human healthspan and lifespan through different mechanisms. These mechanisms include mitigating the harmful effects of reactive oxygen species.
and supporting the health and function of mitochondria. The longevity molecules were carefully selected based on their direct or indirect involvement in processes supporting cell viability and protein expression.

Antibody structure

The canonical antibody belongs to the IgG class of antibodies, which are heterotetrameric proteins (Figure 2). They consist of two different polypeptide chains, two identical heavy chains (50 kDa), and two identical light chains (25 kDa), covalently linked by disulfide bridges, forming a symmetrical Y-shaped structure. The heavy chain is composed of three constant domains (CH1-3). CH1 and CH2 are connected to CH3 by a short linker region called the hinge region, followed by one variable domain called the variable heavy domain (VH). The two heavy chains of the IgG are linked together by two disulfide bridges in their hinge regions. The CH3 regions are also linked to the light chains by a third disulfide bridge. Each light chain consists of one constant domain (CL) and one variable domain (VL). Each "arm" of the IgG antibody comprises the VH and CH3 of the heavy chain combined with CL and VL of the light chain. This is known as the fragment antigen binding (Fab) region and can be cleaved from the IgG to create a Fab fragment (Figure 2B). The top portion of each antibody "arm," known as the VH and VL domains, comprises the Fv (fragment, variable) region, which contains the target (antigen) binding sites (paratopes) that bind to a specific region of their antigen called an epitope. The paratopes consist of six hypervariable amino acid loops called complementary determining regions (CDRs), with three located in the heavy chain (VHCDR1-3) and three in the VL (VLCDR1-3). By recombinantly linking the VH and VL regions using a short linker, a single chain fragment variable (scFv) can be created (Figure 2C). The Fc (fragment, crystallizable) region comprises the CH2 and CH3 domains of both heavy chains. The Fc region modulates immune system responses as it can be bound by Fc receptors on immune cells. Furthermore, the Fc region can also be bound by the neonatal Fc receptor on blood cells, which significantly increases the half-life of antibodies in the bloodstream.
Antibody engineering and binding properties

Recombinant gene technology has enabled the engineering of antibody constructs with practically limitless variations. These constructs can be broadly classified into two categories: IgG-like antibody constructs, which retain the Y-shape of IgGs\textsuperscript{37}, and non-IgG-like antibody constructs, which typically consist of single or multiple antigen-specific fragments or small peptides\textsuperscript{32}.

Two essential and interconnected properties of antibodies are affinity and avidity. Affinity refers to the strength of the interaction between a paratope of an antibody and its epitope, while avidity represents the combined binding strength of multiple binding interactions between an antibody and its target. Specificity, in the context of antibody binding, has two different meanings. Firstly, it describes how well an antibody can bind its intended target without binding other targets, also known as the degree of cross-reactivity. Secondly, it is used to describe the number of epitopes that an antibody recognizes. The latter definition will be used hereafter. Binding valency is another crucial property that refers to how many binding interactions an antibody is capable of, which depends on the number of paratopes it has. The specificity and valency of an antibody both influence its binding avidity. Binding avidity can consist of an antibody binding two or more of the same epitopes on one target molecule. However, for bispecific and multispecific antibody constructs, it can also consist of binding two or more different epitopes on one target molecule\textsuperscript{38}.

Figure 2. Schematics of IgG antibody and antibody fragment structures. (A). Full-size IgG1; VL: variable light chain, CL: constant light chain, VH: variable heavy chain, CH: constant heavy chain, Fab: antigen-binding fragment, Fc region: constant Fc-receptor binding domain. (B). Fab fragment. (C). Single chain variable fragment.
A typical IgG antibody has two identical paratopes and can bind to two identical epitopes on the same antigen, a process known as bivalent binding. However, antibody constructs can be engineered to have different numbers of paratopes, making them monovalent, bispecific, or multispecific binders. The specificity of antibodies and antibody constructs is determined by the number of epitopes they can recognize, and a typical IgG with two identical paratopes is, therefore, monospecific (Figure 3A). In comparison, antibody constructs may have two distinct paratopes (bispecific) or more than two distinct paratopes (multispecific). IgG-like antibody constructs may be engineered to be bispecific by modifying one of the two binding arms so that each binds different epitopes, such as an asymmetrical knob-in-hole IgG\(^39\) (Figure 3A), or by adding one or more scFvs specific to a secondary epitope to an IgG, such as an IgG-scFv\(^40,41\) (Figure 2B). In the first case, the antibody construct would be a bispecific monovalent binder. In the second case, it might bind with varying specificity and valency depending on the number of scFvs added and their locations. IgG-like and non-IgG-like antibody constructs (Figure 3C) may be engineered to be monovalent, bivalent, or multivalent binders and monospecific, bispecific, or multispecific.

Figure 3. Examples of engineered antibody constructs. (A). Knob-into-hole IgG, with CH3-domains engineered to promote hetero-dimerization. (B). IgG-scFv, bispecific construct with scFvs specific to a second epitope linked to the C-terminal end of the IgG light chain. (C). di-scFv, a non-IgG-like monovalently binding bispecific antibody construct.

This customizability of antibody constructs is a great asset, and by modifying binding properties, size, half-life, etc., it is possible to engineer new antibody therapeutics with properties adapted to the specific needs of the drug. However, as previously mentioned, these abovementioned strengths of antibody
therapeutics also present a challenge as they are much larger than small molecule drugs\textsuperscript{42,43}. Large molecules have poor diffusion in the body, and in most cases, they cannot cross barriers, preventing them from reaching targets inside of cells or compartments of the body that are protected by barriers such as the aforementioned BBB\textsuperscript{5}.

The challenge of crossing the blood-brain barrier (BBB)
The blood-brain barrier (BBB) is one of the most tightly regulated interfaces in the body, ensuring brain homeostasis and preventing substances in the blood from freely entering the brain (Figure 4). Small hydrophilic molecules (less than 400 Da and forming fewer than eight hydrogen bonds) can diffuse through the tight junctions between the brain endothelial cells (BECs). In contrast, small lipophilic molecules (such as small molecule drugs) can diffuse through the BECs\textsuperscript{44–49}. However, when injecting antibodies intravenously, only a tiny fraction (0.009\textpm0.001\%) of the dose reaches the brain parenchyma\textsuperscript{50}. As a result, researchers have studied the structure, function, and transport mechanisms of the BBB, which have been extensively reviewed in the literature\textsuperscript{46,48,49,51,52}. The BBB consists of endothelial cells BECs supported by pericytes (PCs), astrocytes, and extracellular matrix (EM) structures, including basal lamina and glycocalyx.

**Figure 4.** Schematic of the BBB.

The BECs of the BBB provide the foundation for the barrier with their key features, namely, intra-endothelial tight junctions between neighboring BECs,
lack of fenestration, lack of pinocytic activity, enzymatic regulation, and specialized polarized transport systems. The tight junctions of the BECs make them closely connected and effectively restrict the unregulated paracellular passage of substances into the brain. The lack of fenestration in the BEC endothelium also prevents the exchange of molecules between the brain and the blood\textsuperscript{53}. The lack of pinocytic activity prevents the unregulated entry of substances, and the presence of enzymatic regulation also contributes to this regulation by the presence of drug-metabolizing enzymes, e.g., CYP450. The polarized transport systems include P-glycoprotein, an efflux pump that actively pumps out substances within BECs back to the blood circulation\textsuperscript{53,54}.

The BECs are enveloped by PCs and astrocytic end-feet, where they both provide the support necessary for maintaining the homeostasis and function of the BBB. PCs contribute by exchanging essential molecules such as second messengers, ions, and metabolites, supporting microvascular stability, regulating cerebral blood flow, and forming the basal lamina\textsuperscript{55}. Astrocytes are vital glial cells extending cellular processes to neurons and BECs. The astrocytic end-feet almost completely envelop the BBB and support BBB integrity and function in several ways by, e.g., providing several growth factors that are necessary for the maintenance of BECs' aforementioned tight junctions, enzymatic regulation, and polarized transport systems, as well as the formation of the basal lamina\textsuperscript{55}.

The glycocalyx and the basal lamina provide an additional barrier between the blood circulation and the brain, with the glycocalyx lining the luminal side of BECs, thus making it the first point of contact for any substance in blood circulation. The constituents of the basal lamina and glycocalyx include structural proteins such as collagens and laminins, hyaluronic acid, glycolipids, and glycoproteins such as heparan sulfate (HS) and HS proteoglycans (HSPGs).

Heparan sulfate (HS) and HS proteoglycans (HSPGs)

HS is a type of linear glycosaminoglycan polymer that was first believed to be a less sulfated byproduct of heparin synthesis. Though HS was shown to be a distinct molecular entity over 60 years ago\textsuperscript{56}, the elucidation of its structure has been a slow and painstaking process due to its complexity. HS is a family of related polymers rather than a single compound due to its structural heterogeneity and variability. Unlike heparin, which is found only in mast cells (a type of immune cell), HS is produced by all cells in the body and occurs in the form of HSPGs on cell surfaces and in the ECM\textsuperscript{57}. Heparin and HS are made of repeating disaccharide units containing glucosamine and uronic acid. The initial unit has glucuronic acid linked to N-acetylg glucosamine. Potential modifications occur sequentially, starting with N-deacetyla-
tion and N-sulfation of glucosamine residues. Then, epimerization of glucuronic acid to iduronic acid and O-sulfation occurs. Finally, O-sulfonation occurs at the C-3 of the glucosamine\textsuperscript{58}. Each of the biosynthetic reactions in this pathway is dependent to some extent on the previous modification, as the products of one step can often act as substrates for subsequent steps. It is worth noting that each of these biosynthetic modification steps is unlikely to proceed to completion. Thus, the resulting chain lengths can be of different lengths and can be differentially modified in various regions, which accounts for a significant component of the structural heterogeneity observed in these molecules\textsuperscript{59,60}. Heparin has an average of 2.7 sulfate groups per disaccharide, while HS has at least 1 sulfate group per disaccharide\textsuperscript{61}. HS contains all the structural variations found in heparin, but it has a higher frequency of minor sequence variations, is more structurally heterogeneous, and has longer chain lengths, \textasciitilde30 kDa, compared to \textasciitilde15 kDa for heparin\textsuperscript{58}.

HS chains are usually found in tissues covalently attached to core proteins in PG structures\textsuperscript{62}, where one PG molecule can have several HS chains attached to it. The different HSPGs are categorized into four major groups that differ in their core protein structures. Among these groups, two families of HSPGs are associated with the plasma membrane of cells, namely transmembrane syndecans and glycosylphosphatidylinositol-anchored glypicans. A third group includes several secreted forms, which are integral components of the ECM, such as perlecan, agrin, and collagen XVIII. Although HS chains synthesized by a given cell are often similar, they may be linked to different core proteins\textsuperscript{63}. Therefore, the general concept is that HS structure is cell-specific but not PG type-specific\textsuperscript{55}. However, as our capacity to sequence chains from specific PG attachment sites grows, this general concept may shift. New data in the field suggests that there may be not only PG-type-specific chains but even attachment site-specific chains in regard to the specific core PG protein\textsuperscript{64}. Further, HS structure can also vary within the same cell type due to chain-modifying processes, including, for example, cleavage by the enzyme heparinase during inflammation\textsuperscript{65}.

These abovementioned structural properties of HS allow HSPGs to provide a wide range of functions, including regulation of endocytosis, regulation of receptor trafficking, and being co-receptors for a wide range of ligands\textsuperscript{5,55,66–69}. Specifically, cell surface HS is believed to assist cellular uptake of large molecules by functioning as co-receptors and presenting the ligand to its receptor and/or stabilizing receptor-ligand interactions\textsuperscript{67,70–72}. These cellular uptake-promoting functions of HS prompted us to investigate HS binding as a potential factor influencing the brain uptake of BBB shuttle scFv8D3 (\textbf{Paper II}).
As the BBB presents a considerable challenge for the brain delivery of antibody therapeutics, researchers have investigated strategies that target natural transport mechanisms capable of transporting large molecules into the brain.

**Transport through the blood-brain barrier (BBB)**

Small molecules that cannot diffuse through the BBB (such as glucose, amino acids, vitamins, etc) are transported via active transport proteins or specialized solute carrier transporters. Conversely, large molecules are transported via vesicular transport mechanisms\(^5\) (Figure 5), starting with the cellular uptake by inward budding of the cell membrane, resulting in the formation of endocytic vesicles containing the ingested material, referred to as endocytosis. Endocytosis may occur by different mechanisms involving caveolar vesicles, pinocytic activity, or clathrin-coated vesicles. Caveolar endocytosis and pinocytic activity occur independently of interaction between the cargo and the endocytic vesicle. In contrast, adsorptive-mediated endocytosis (AME) and RME are both believed to be dependent on interactions between the cargo and the vesicle, and both have been reported to occur at clathrin-coated pits at the BBB\(^5,40,69\). AME is characterized by unspecific charge-dependent interaction between cationically charged cargo and anionically charged HS chains of HSPGs, and HSPGs are known to be present at the surface of clathrin-coated pits which are frequently formed at the cell surface of BECs\(^69\). The subsequent transcytosis of cargo endocytosed via AME, i.e., adsorptive mediated transcytosis (AMT), is currently poorly understood, and AMT has been reported to occur both with and without clathrin-coated vesicles\(^69\). In the case of RME, endocytosis is known to occur through clathrin-coated vesicles forming at clathrin-coated pits in the cell membrane, which is an exceedingly regulated and intricate mechanism involving over 50 different proteins\(^73\). Receptor-ligand complexes are endocytosed into cells, which is the first step in receptor-mediated transcytosis (RMT). In RMT, the endocytosed vesicles are intracellularly sorted via sorting endosomes to the basolateral side of the endothelium, where the vesicles fuse with the membrane, releasing their cargo. Alternatively, the vesicle may be released as exosomal vesicles (exosomes)\(^5\). Exosomes are formed by the inward budding of endosomes, creating intraluminal vesicles (ILVs) within multivesicular bodies (MVBs), which are then released as exosomes\(^74\).
Different approaches utilizing the AMT and RMT transport mechanisms have been investigated to deliver antibody therapeutics into the brain. Cationization of, e.g., Fab regions has been tried to induce AMT. However, this method has been shown to be problematic due to the unspecific cationic interaction, which led to side effects such as toxicity, decreased brain uptake, and distribution to peripheral tissues\textsuperscript{69,75–77}. In comparison, targeting RMT-capable receptors enriched at the BBB has been shown to be more effective for delivering antibody therapeutics into the brain and increasing brain-specificity\textsuperscript{5,45}. This brain delivery strategy has been referred to as the molecular Trojan Horse" strategy\textsuperscript{5,46,78,79}.

Receptor targets for the molecular Trojan horse strategy

To execute the molecular trojan horse strategy, researchers have investigated several receptor targets, such as the insulin receptor\textsuperscript{80–82}, transmembrane protein 30A\textsuperscript{83,84}, the low-density lipoprotein receptor-related protein 1\textsuperscript{85,86}, CD98 heavy chain (a heavy chain of heterodimeric amino-acid transporters)\textsuperscript{87–89}, and the transferrin receptor (TfR), which is the most widely studied target\textsuperscript{46,90}. TfR-targeting has been extensively studied in multiple species, including mice\textsuperscript{21,40,41,91–100}, rats\textsuperscript{101–104}, and non-human primates\textsuperscript{96,105,106}, and its use has even brought several antibody therapeutics targeting brain disorders to clinical trials\textsuperscript{107–109}.

Examples of TfR-binding antibody therapeutics include pabinafusp alfa, which is a drug consisting of recombinant iduronate-2-sulfatase enzyme fused to a TfR-binding IgG antibody, developed as a replacement therapy by JCR
Pharmaceuticals for the treatment of the lysosomal storage disorder Hunter syndrome\textsuperscript{107–109}. Pabinafusp alfa was approved in Japan in March 2021\textsuperscript{110}, and the drug is currently in the global phase III STARLIGHT study (NCT04573023) in Europe, the USA, and Brazil. In 2020, Denali Therapeutics published their BBB Transport Vehicle (TV)-technology, which is an engineered Fc (fragment crystallisable) containing a TfR-binding domain capable of delivering biological drugs into the brain\textsuperscript{96} with three antibody therapeutic drugs (DNL310, DNL593, and DNL919) using this TV technology currently in clinical trials. Like Pabinafusp alfa, the DNL310 drug was developed as a replacement therapy for Hunter's syndrome\textsuperscript{111,112}. DNL310 is currently being tested in the phase I/II trial NCT04251026 and the phase II/III COMPASS trial (NCT05371613). Data recently presented at WORLDSymposium\textsuperscript{TM} 2023 from the I/II trial indicates improvement in the participants' clinical symptoms\textsuperscript{113}. The second drug, DNL593, was developed as a replacement therapy for frontotemporal dementia with GRN mutations where progranulin is fused to the TV\textsuperscript{114}, and it is currently in a phase I/II trial (NCT05262023). The third drug, DNL919, was developed to treat Alzheimer's disease (AD) and consists of the TV fused to an F(ab')\textsubscript{2} of an anti-TREM2 antibody\textsuperscript{115}, which is currently in a phase I trial (NCT05450549). Roche has also developed a BBB shuttle, a TfR-binding Fab\textsuperscript{94,116}. By fusing this shuttle to an anti-amyloid beta (A\textbeta) antibody, they created the BBB-penetrating AD-drug trontinemab, which is currently being tested in the phase I/II trial (NCT04639050). Additionally, Roche has developed an anti-CD20 antibody fused with their BBB shuttle drug (RG6035 or RO7121932) for the treatment of multiple sclerosis by immunosuppression, and this antibody therapeutic is currently in a phase I clinical trial (NCT05704361).

Although the exact molecular mechanisms enabling brain delivery of antibody therapeutics by targeting TfR are still unknown, the results of the aforementioned studies and trials suggest that it is a promising approach.

Transcytosis of transferrin (Tf) via transferrin receptor 1(TfR)

TfR is a transmembrane glycoprotein that is highly enriched at the surface of the BBB. It is a homodimeric protein responsible for transporting iron into the brain via its natural ligand, transferrin (Tf)\textsuperscript{117}. TfRs subunits consist of an intracellular domain located at the N-terminus, a transmembrane domain, and an extracellular domain connected to the transmembrane domain by a stalk domain. The extracellular domain has a butterfly-wing shape and is made up of three subdomains, including a protease-like domain, an apical domain, and a helical domain (Figure 6A) where the Tf ligand binds (Figure 6B)\textsuperscript{118,119}. Tf
is a bilobular glycoprotein consisting of a single polypeptide chain with one ferric Fe\(^{3+}\) ion binding site on each lobe. Its affinity to iron is pH-dependent, and though it is exceptionally high (\(K_D = 10^{-13}\) nM) at the physiological pH of 7.4, it is reversible\(^{120-122}\). Tf exists in three different forms: both binding sites unoccupied (apoTf), one binding site occupied (mono-ferric Tf), and both sites occupied, i.e., diferric Tf (holoTf)\(^{123-126}\).

Figure 6. TfR and holoTf. (A). The extracellular domain of the TfR homodimer (PDB: 1SUV) with one subunit colored in grey (left) and the other colored by domain (right)\(^{127}\). (B). The TfR homodimer (grey) can bind two holoTf molecules (blue) per subunit (PDB: 1SUV).

At physiological pH, holoTf has the highest affinity to TfR (\(K_D \sim 4\) nM), and mono-ferric Tf follows with an affinity of 32-36 nM. In contrast, apoTf's affinity is so low that it likely does not bind TfR at all. Tf undergoes structural changes upon binding with iron, which leads to its selective recognition by TfR\(^{128}\). The complex of holoTf and TfR is then internalized through clathrin-coated pits\(^{73,129}\), but its fate after endocytosis and the process of iron transport into the brain is a topic of controversy as hypotheses regarding the molecular mechanisms at work are based primarily on studies in other cell types, such as red blood cells (erythrocytes). The first of the two hypotheses regarding the fate of Tf and iron at the BBB is based mainly on mechanisms occurring in erythrocytes, which I will therefore refer to as the erythrocyte-based hypothesis. The erythrocyte-based hypothesis stipulates that iron bound to holoTf may dissociate within the early endosome due to the reducing activity of the endosomal metalloreductase protein STEAP3, converting ferric iron (Fe\(^{3+}\)) to ferrous iron (Fe\(^{2+}\)). This allows the divalent metal transporter 1 (DMT1) to transport iron into the cytosol. In the cytosol, the iron can be metabolized, stored by iron storage proteins like ferritin, or exported out into the brain via the iron transporter protein ferroportin. Meanwhile, apoTf may remain in complex with TfR due to its increased affinity within the acidic environment of the early endosome. The apoTF-TfR complex is then suggested to either be
recycled back to the apical membrane of the BECs or be sorted to the basolateral membrane and be transcytosed into the brain parenchyma, where apoTf can dissociate from TfR due to the physiological pH\textsuperscript{46,125,130}. The second hypothesis stipulates that holoTf is transcytosed in an unaltered fashion through the BECs via sorting tubules\textsuperscript{138} into the brain parenchyma, where it is stipulated to dissociate from TfR despite having high affinity to TfR at physiological pH. I will refer to the second hypothesis as the holoTf-transport hypothesis. The holoTf-transport hypothesis has been criticized for being unable to solely account for brain iron uptake and homeostasis. For example, findings in rats have shown that much more iron than Tf is transported into the brain\textsuperscript{131–133}, and loss-of-function mutations in the Fe\textsuperscript{2+} iron pump DMT1 decrease brain iron concentrations in rats\textsuperscript{134}. Furthermore, the holoTf transport hypothesis alone does not explain how BECs obtain the iron required for their functioning. In addition, there is evidence from in vitro studies that the BECs cells of the BBB play a role in regulating iron uptake and maintaining iron balance in the brain\textsuperscript{135,136}. As these two theories are not mutually exclusive, both pathways may occur at the BBB\textsuperscript{46}, which has been reported to be the case in an in vitro BBB model\textsuperscript{137}. In the case of TfR-binding antibody therapeutics, the currently accepted brain delivery mechanism stipulates sorting of endocytosed vesicles to the basolateral BEC membrane, followed by cargo release into the brain parenchyma\textsuperscript{46}. In Paper IV, we investigated if fusing holoTf to the TfR-binding arm of an antibody therapeutic could improve its in vitro BBB transcytosis.
Figure 7. Hypotheses of iron uptake and transport of iron and Tf at the BBB. Fe$^{3+}$ bound to holoTf is endocytosed by RME after binding to TfR. The first hypothesis stipulates that the holoTf-TfR complex is sorted to an acidified endosome, while according to the second hypothesis, the complex is transported unaltered via sorting tubules to the basolateral membrane, where holoTf is believed to dissociate from TfR despite having high affinity to TfR at physiological pH (holoTf transcytosis). In contrast, according to the first hypothesis, the low pH causes Fe$^{3+}$ to dissociate from Tf and subsequently become reduced to Fe$^{2+}$ by the endosomal metalloreductase protein STEAP3. Fe$^{2+}$ is then transported out into the cytosol by the transporter DMT1 and bound by iron storage proteins, e.g., ferritin. Finally, Fe$^{2+}$ can be exported (Fe$^{2+}$ export) into the brain via the iron transporter protein ferroportin (or stored intracellularly or metabolized). The remaining non-iron loaded apoTf has a high affinity to TfR at acidic pH and likely remains bound to TfR until it is recycled back to the apical membrane and released there (apoTf recycling), released at the basolateral membrane (apoTf transcytosis), or sorted to a lysosome for degradation.
Although several TfR-binders have been developed to implement the molecular Trojan horse strategy, most have not performed as expected. Different obstacles, such as the properties of the BBB, elimination in blood, binding to off-target cells expressing TfR such as blood cells, and especially the properties of the TfR-binders themselves, need to be considered to achieve effective brain delivery. To this end, researchers have been investigating the specific properties of TfR-binders that can promote successful brain delivery.

Properties of TfR-binders influencing brain delivery

How antibodies bind to TfR has an important impact on their ability to pass through BBB, where binding affinity and valency appear to play critical roles. Research suggests that monovalent binding is more effective at facilitating brain uptake compared to bivalent binding. It has been proposed that one reason is that antibodies binding TfR bivalently may cause cross-linking of TfR receptors on the cell, promoting lysosomal degradation of the antibody-receptor complexes instead of transcytosis. This is believed to be particularly likely at high antibody concentrations. Moreover, the avidity effect of binding TfR with multiple domains simultaneously causes bivalent binders to have slower TfR dissociation rates, thereby increasing their affinity. Too slow TfR dissociation rate and too high affinity have also been proposed to promote lysosomal degradation and to cause TfR down-regulation, leading to decreased brain delivery of bivalently binding TfR antibodies, as well as for monovalently binding TfR antibodies. Therefore, the lack of binding avidity in monovalent binding might also be beneficial due to faster TfR dissociation, possibly leading to greater transcytosis. The affinity of TfR binders must be low enough to enable adequate dissociation from TfR. Nevertheless, if it is too low, antibodies may not be able to sufficiently associate with TfR at the BBB surface, preventing endocytosis and subsequent transcytosis into the brain. TfR affinity also strongly influences blood half-life, which is a third important property of TfR binders. The blood half-life of anti-TfR antibodies is inversely related to their TfR affinity. Therefore, TfR binders with moderate TfR affinity have a longer half-life, increasing their brain exposure over time, allowing them to achieve higher brain concentrations. Finally, pH-dependent TfR affinity has been put forth as a mechanism that enables antibodies to dissociate from TfR during the acidification of late-endosomes (LEs) where the pH decreases to approximately 5.5, whereby these BBB shuttles are thought to escape lysosomal degradation.
Based on these aforementioned properties, possible transcytosis mechanisms of TfR-binding BBB shuttles (Figure 8) are summarized in the following manner:

1) Low-to-moderate affinity, monovalently binding (and possibly pH-dependent) TfR-binding BBB shuttles present in the blood circulation bind to TfR in clathrin-coated pits on the surface of the BBB. They are then endocytosed in clathrin-coated vesicles via RME.

2) The low-to-moderate affinity and monovalently binding BBB shuttles in complex with TfR are sorted LEs, whereupon the BBB shuttles dissociate from TfR and escape lysosomal degradation. In contrast, high affinity bivalent TfR binders either cross-link many of the receptors at the BEC cell surface and/or fail to dissociate from TfR and are therefore sorted into lysosomes where they are degraded.

3) The BBB shuttles are then sorted to the basolateral membrane by sorting tubules that, upon membrane fusion, release free BBB shuttles. Alternatively, the BBB shuttles become part of ILVs within an MVB, which are released as exosomes after the MVB fuses with the basolateral membrane.
Figure 8. Schematic of possible transcytosis mechanisms of TfR-binding BBB shuttles. The fates of BBB shuttles depend on their binding valency and affinity. Low-to-moderate affinity, monovalently binding BBB shuttles are sorted into LEs or MVBs. In the LEs, the BBB shuttles dissociate from TfR and are trancytosed via sorting tubules that fuse with the basolateral membrane, where the BBB shuttles are released into the brain parenchyma. Alternatively, the shuttles become part of ILVs within MVBs, which are sorted to the basolateral membrane. After the MVBs fuse with the membrane, the ILVs are released as exosomes. In contrast, bivalent and high affinity BBB shuttles that fail to dissociate from the TfR receptor or that cross-link several TfR receptors at the basolateral surface are preferentially sorted to lysosomes where the shuttle-receptor complexes are degraded. This fate seems to be especially prevalent at therapeutic dosages, whereas, at low doses, bivalent and high affinity BBB shuttles can cross the BBB. In the case of successful transcytosis, TfR receptors are believed to be recycled back to the apical membrane of the BECs and participate in successive RMT events.
Reducing TfR affinity of monovalent TfR binders to improve brain delivery

Reducing the affinity of high affinity anti-TfR antibodies by introducing point mutations in their CDRs has been shown to improve brain delivery of both bivalently and monovalently binding TfR-antibodies\textsuperscript{41,88,99,147}.

In 2013, Couch and colleagues conducted a study investigating the impact of reducing the affinity of an asymmetric IgG antibody that binds to TfR monovalently. They generated three monovalent affinity mutants and observed that the plasma half-life of the affinity mutants was significantly extended compared to the original antibody. The lowest affinity mutant had a half-life almost as long as a control IgG antibody. The researchers also studied the brain concentration of the mutants over ten days in vivo and observed that the lowest affinity mutant exhibited poor brain concentrations throughout the study period. In contrast, the medium affinity mutant had the highest brain concentration over time, which coincided with its prolonged half-life. These results suggest that the affinity of the lowest affinity mutant was reduced too much\textsuperscript{144}, which prevented it from adequately associating with TfR\textsuperscript{105,144}. Building on this work, Bien-Ly et al. investigated the effect of affinity on TfR trafficking and brain delivery for monovalent TfR binders. In the study, Bien-Ly et al. compared the same, relatively high affinity, asymmetric monovalent TfR-binder created in 2013 by Couch et al. to the medium affinity mutant and reported that the high affinity binder colocalized with lysosomal markers during in vitro live imaging and caused cortical TfR degradation in vivo\textsuperscript{142}. In contrast, the medium affinity mutant avoided these effects and achieved higher brain concentrations\textsuperscript{142}.

In Paper III, we apply the strategy of reducing the TfR affinity of a single-chain monovalent TfR binder to prolong its blood half-life and enhance brain delivery.

Monovalent TfR antibody design

A common feature of antibody therapeutics utilizing TfR-RMT to reach the brain is binding TfR monovalently. One previously mentioned example is Denalis TV technology, where the TfR paratope is situated in one of the heavy chains of the Fc-fragment to which intra-brain target binding paratopes may be fused\textsuperscript{96}. Another is asymmetric IgG-like antibodies where one of the arms binds to TfR\textsuperscript{41}, and the other arm binds a therapeutic target, e.g., amyloid beta in the case of AD\textsuperscript{47,98}. Both of these examples utilize a technique called knob-into-hole commonly employed for producing asymmetric antibodies, where
modifications in the Fc chain prevent the pairing of identical heavy chains and thus partly inhibit the formation of unwanted homodimers\textsuperscript{148}. However, one downside with the knob-into-hole approach is that the yields are significantly decreased due to unwanted homodimeric pairings\textsuperscript{149}. Recently, in our group, we have created a single-chain Fc (scFc) by adding a linker that connects the two chains of the mouse IgG2c Fc region and mutating the most C-terminal of the cysteine residues in the hinge region to remove a disulfide bond and thereby increasing the construct's flexibility (Figure 9B). To this scFc, a TfR-binding scFv has been added, creating a single-chain monovalent BBB shuttle, which can be used as a base for creating BBB-penetrating antibody therapeutics (Figure 9C). The design of this antibody construct makes it easier to produce because it is a single chain and, therefore, does not require Knob-into-hole pairing. It also ensures monovalent TfR binding while also providing extended blood half-life and stability imparted by the Fc-region\textsuperscript{150}

Figure 9. Schematic of scFc and scFc-scFv8D3 construct designs. (A) Full-size murine IgG2c with three cysteine bridges present in the hinge domain. (B) The single chain fragment constant (scFc) is made by linking two chains of the mouse IgG2c Fc region into a single chain by a short flexible linker. The cysteine residues closest to the C-terminal-s in the hinge region were mutated to remove one of the disulfide bridges, which increases the construct flexibility. (C) A monovalently binding BBB shuttle is created by linking a TfR binding single chain fragment variable (scFv) of the antibody 8D3 (scFv8D3) to the hinge region of the scFc platform.

The monovalent BBB shuttle scFv8D3

The high-affinity anti-TfR antibody 8D3\textsuperscript{95,151} binds to an epitope on the extracellular apical domain of the murine TfR (mTfR), distinct from that of transferrin\textsuperscript{46}, which prevents it from disturbing iron transport into the brain. 8D3
has been used extensively in different formats, in our lab and by others. By using an scFv format of 8D3 (scFv8D3) or the scFc-scFv8D3 format (Figure 9C), one can ensure monovalent TfR-binding and both of these formats have been used in the papers included in this thesis.
Methods

Protein design

Recombinant DNA technology has revolutionized the development of antibody therapeutics. This technology allows for the modification of protein drugs in various ways, enabling tailored target engagement(s). Once a protein sequence is determined, it can be reverse-translated into a nucleotide sequence and inserted into DNA plasmids. Furthermore, this technology has made it possible to combine more than one protein, peptide, or domain in the same design, forming fusion proteins, bispecific/multispecific proteins, and the generation of small fragments such as scFvs. In Paper II, the BBB shuttle scFv8D3 was used. The scFv (Figure 2C) consists of the heavy and light chain variable domains of the 8D3 antibody linked together with a glycine-serine (G4S) linker and a 2 x Strep II-tags linked to the C-terminal end via an in-house designed linker (APGSGTGSAPG) to aid downstream purification and detection. The in-house designed linker has proline residues at both ends to disrupt alpha-helix formations, and a threonine residue introduced in the middle decreases the risk of developing anti-drug antibodies. To introduce or remove HS-binding sites on the surface of scFv8D3, a homology-modelled structure was generated by using the automated protein structure homology-modelling server SWISS-MODEL, based on the primary amino acid structure of scFv8D3. Proteins bind to negatively charged HS using positively charged HS binding sites consisting of amino acids like arginine, lysine, or histidine spaced approximately 5-10Å or 20Å apart. Canonical HS binding sites (HS binding motifs) consist of linear peptide regions that occur in patterns such as XBBXBX, BBBXXBB, or BBXXBB, where B denotes positively charged amino acids. Positively charged amino acids that are distant in sequence can be brought together spatially due to protein folding. For instance, antithrombin III has a higher order HS binding site (HS binding cluster), including a linearly contiguous domain and a sequence-remote, positively charged amino acid.
In Paper III, the scFc (Figure 9B) was created in our lab prior to the thesis work by linking two mouse IgG2c Fc region chains into a single chain by a short flexible linker (P, followed by G4S). The cysteine residues closest to the C-terminal-s in the hinge region were mutated to remove one of the disulfide bridges, which increases the construct flexibility. To the N-terminal end of scFc, the scFv8D3 was fused via the in-house designed linker, creating the single chain monovalent BBB shuttle scFc-scFv8D3 (Figure 9C). To decrease the TfR affinity of scFv8D3, point mutations were introduced based on theoretical functional group analysis and in silico protein-protein docking analysis using the software ezPPDock and AbAdapt.

In Paper IV, a fusion antibody based on the IgG-scFv bispecific antibody (Figure 3B) construct RmAb158-scFv8D3 was generated by fusing holoTf to the C-terminal end of the antibody’s light chain via the in-house designed linker, creating the RmAb158-scFv8D3-holoTF fusion antibody.

Protein expression and purification

Each design's complete sequence was integrated into a single pcDNA3.4 expression vector (Papers II and III). However, for antibody expression, the heavy and light chain plasmids were cloned into two separate vectors (Papers I and IV). Signal peptides originating from Tf were added to the N-terminal end of each design to ensure that the proteins were trafficked through the secretory pathway and released into the cell media. The signal peptides are 19-20 amino acids long and are not part of the final protein, as they are cleaved off during translocation.

The cloning of the designs into the expression vector was performed by ThermoFisher GeneArt, after which the plasmid DNA was amplified using E. coli Top10 cells and purified using a commercially available maxiprep kit.

For the protein expression, mammalian cells were used to ensure proper protein folding and PTMs. Specifically, Expi293 cells were used, which are HEK293 cells adapted to grow in suspension at high density and therefore capable of producing substantially higher recombinant protein yields per transfection volume. In Paper I, we detailed our updated TGE protocol and developed a small-scale version of said protocol.

For protein purification, clarified and filtered cell media supernatant was loaded on different binding columns using an Äkta system. Antibodies con-
taining Fc parts from the clarified supernatant were purified with HiTrap Protein G columns. Depending on which tag is included in the design, either HisTag or StrepTrap columns were used for proteins lacking the Fc domain.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE can be used to separate proteins by their mass-to-charge ratio. SDS's surfactant and anionic properties allow it to cover proteins and mask their intrinsic charge by making them negatively charged based on their size, as one SDS molecule binds for every two amino acids in the proteins. The concentration of polyacrylamide in the gels used in SDS-PAGE determines the gel’s pore size, and smaller proteins move through the gel faster due to being less hindered than large proteins in their passage through the gel pores. Applying a current allows the anionized proteins to travel through the gel toward the cathode at a speed dependent only on size. Afterward, the gel can be stained using coomassie brilliant blue dye, which colors the proteins in the gel and allows for assessment of the size and purity of the separated proteins.

Enzyme-linked immunosorbent assay (ELISA)
ELISA is a technique that relies on interactions between an antigen on the protein of interest and antibodies. The method is very sensitive, typically having a detection range between 0.1 to 1 fmole (or 0.01 to 0.1 ng). The basic setup involves a primary antibody that binds to the protein of interest, which has been immobilized in a multiwell plate, a secondary enzyme-linked antibody that binds to the primary antibody, and a substrate that undergoes hydrolysis and induces emission of a detectable signal that can be measured by, e.g., spectrophotometer. However, many different types of ELISA setups can be used; direct and indirect ELISA, sandwich ELISA, and inhibition ELISA are the most commonly used setups.

Quantification ELISA
To quantify the expression levels of the antibody in Paper I, unfiltered and unpurified cell media was analyzed with a previously published sandwich ELISA-setup. The advantage of sandwich ELISA setups is that they enables one to selectively detect the antigen of interest out of a complex sample such as a biological matrix (e.g. cell media or tissue homogenate), removing
the need to purify the antigen before analysing. Additionally, the setup is extraordinary sensitive, allowing for the quantification of picomolar concentrations of antibody constructs\textsuperscript{150,168}.

Transferrin receptor (TfR) ELISA
To assess the binding of antibody constructs in Papers II and III, a previously published indirect ELISA setup was used\textsuperscript{169}. In short, serial dilutions of the antibody constructs were added to ELISA plates coated with the extracellular domain of mTfR and detected. The advantage of this setup is its simplicity and its capacity to determine if an antibody construct binds to its intended target. In Paper IV, an inhibition ELISA setup was used. Briefly, antibody constructs and controls were first incubated with serial dilutions of mTfR in a non-binding plate to allow the binding to occur unhindered, after which the mixture was added to a plate coated with a TfR binder, where the mTfR available from the mixture could bind to the coat and be detected. Similarly to the indirect ELISA, this setup can be used to determine if the antibody construct binds to TfR. It was used for antibody constructs containing holoTf-domains as we have observed that Tf binds poorly to immobilized mTfR.

Thermal shift assay
The stability of the antibody constructs in Paper II was analyzed by a thermal shift assay using a Tycho nt.6 instrument, which measures fluorescence at the 330 and 350-nanometer wavelengths. Thermal shift assays work by measuring the shift of fluorescence intensity of proteins during a temperature gradient between 35°C and 95°C. The temperature gradient induces structural changes in the proteins, which affects the amount of exposed tryptophan and tyrosine residues, which in turn shifts the fluorescence intensity, and major unfolding events can be detected as peaks in the first derivative of the fluorescence ratio 350/330nm which are then called inflection temperatures.

Heparin column liquid chromatography
The heparan sulfate binding capacity of the antibody constructs in Paper II was assessed by heparin column liquid chromatography with an Äkta start instrument. As the binding to the column depends on cationic interactions, charged proteins must be in a buffer with low salt concentration (e.g., 10 mM sodium phosphate buffer) to prevent disruption of binding to the column. The proteins can then be eluted by a linear gradient of NaCl buffer.
In vitro BBB transcytosis assay

In vitro BBB models have been somewhat controversial in the field because of issues with adequately mimicking in vivo properties, such as luminal blood flow\(^ {170}\), but the main critique has been regarding leakiness. Simplified in vitro BBB models with rigorous washing steps included have been developed to circumvent the issue of leakiness, where unbound or leaked antibodies are removed by the washing\(^ {94,146,171}\).

Our In-Cell Trans assay was designed based on these simplified models to evaluate the transcytosis of TfR binding antibody constructs\(^ {172}\), and was used in Paper III and IV. The assay allows for the analysis of antibody therapeutics by adding them to an apical chamber of a cell-coated permeable support membrane. The media in the basolateral chamber is collected and analyzed at various time points, which is referred to as the chase phase. The apical chamber is a static representation of the luminal blood flow seen in the arterial and venous capillaries of the BBB, while the basolateral compartment replicates the abluminal brain environment. If the antibody therapeutic has brain shuttling properties, it should be detectable in the basolateral chamber during the chase phase of the in vitro assay.

Radiochemistry

For the in vivo brain uptake and biodistribution studies in Papers II and III, the antibody constructs were radioactively labeled with iodine-125 (\(^ {125}\)I). The strength of the radiolabeling methodology is its tremendous sensitivity, which allows for measuring minute amounts of radiolabeled samples, such as tracer doses of antibody constructs, making it advantageous for pharmacokinetic studies\(^ {173}\). \(^ {125}\)I has a half-life of 59.5 days, which is suitable for in vivo experiments of up to a few months after administration\(^ {174}\). The signal from the \(^ {125}\)I-antibody constructs the signal can be measured using a gamma counter. Iodine radiolabeling is performed in three steps: The radioiodine is oxidized by an oxidative agent (e.g., Chloramine T), after which the oxidized radioiodine substitutes a hydrogen atom on a phenolic aromatic ring of a tyrosine residue on the protein by a nucleophilic attack. The final step is to stop the reaction and remove excess oxidized radioiodine by adding a reducing agent (e.g., metabisulphite)\(^ {175}\).

In vivo experiments

To assess brain delivery, \(^ {125}\)I-labelled antibody constructs were intravenously injected into the tail vein of wild-type mice (Paper II and III). The mice were
euthanized at various time points through transcardial perfusion with 0.9% NaCl. This step is essential because the concentration of the injected proteins in the blood is high, especially during the early time points. If the mice are not perfused correctly, the accuracy may be affected. By performing the perfusion step before measuring \(^{125}\text{I}\) signal, any background signal originating from the blood is removed, leaving only the radioactive signal bound in the brain. Brains were then dissected and activity was measured using a gamma counter.

To assess the peripheral distribution, various peripheral organs such as the liver, spleen, heart, lung, kidney, pancreas, thyroid, muscle, and bone were also dissected, and the activity was measured (Paper II and III).

We also analyzed the the half-life in blood of antibody constructs (Paper III). To determine the half-life of injected antibody constructs, we took blood samples from the tail vein at different time intervals and measured their activity. Additionally, we determined the concentration of the injected proteins in different blood compartments. To achieve this, we collected terminal blood samples and separated them into supernatant (plasma) and pellet (containing blood cells) through centrifugation, after which we measured the activity of these fractions.

Capillary depletion

In the ex vivo brain uptake studies in Paper II, we supplemented measuring signal from the \(^{125}\text{I}\)-antibody constructs with a gamma counter with two methods that enable distinguishing from which compartment the signal originates, as measuring brain uptake by a gamma counter does not distinguish between radioactive signal inside the brain parenchyma and signal in, or at the cell surface of, the BECs of the BBB. Measuring the \(^{125}\text{I}\) signal in brain samples where the BECs have not been separated from the brain parenchyma may, therefore, overestimate the amount of \(^{125}\text{I}\)-antibody-construct that reaches the brain parenchyma. The first of the two complementary methods is capillary depletion, first published using rat brains\(^{176}\), but later adapted for mouse brains\(^{177}\), which is a qualitative method in which the brain parenchyma and brain capillaries are separated. This separation enables the quantitative estimation of the partitioning of radioactive signal intensity between these two compartments. To separate the brain capillaries from the brain parenchyma, density-gradient centrifugation is applied to a brain homogenate dissolved in a polysaccharide solution, resulting in three fractions: a fatty fraction consisting of, e.g., myelin, a fraction containing parenchymal cells depleted from the microvasculature, and a microvasculature enriched pellet. By measuring the radioactive signal in these separated fractions, the ratio of the signal between different compartments can be obtained, which can be used to assess to what
extent different BBB penetrating compounds reach the brain parenchyma, and thus the efficiency of the brain delivery\textsuperscript{101,178–183}.

Nuclear track emulsion (NTE) autoradiography

The second supplementary method used for the ex vivo brain uptake studies in \textbf{Paper II} was nuclear track emulsion (NTE) autoradiography. NTE allows for the visualization of radiolabeled compounds, this visualization can then be combined with subsequent immunofluorescence and/or immunohistochemistry, and the resulting image overlays can be used to visualize the NTE signal and relevant structures on a microscopic level. A photographic/nuclear emulsion solution containing radiation-sensitive silver halide crystals is used to visualize radiolabeled compounds. The crystals are activated by exposure to ionizing radiation and visible light; therefore, the procedure must be performed in a dark room. After becoming activated, the crystals produce a "latent image" consisting of clusters of silver atoms, halide ions, and free electrons, which may be reduced to metallic silver grains by a photo development process. These silver grains then appear as black puncta corresponding to the area where the radiolabeled compound is localized. One drawback is that the resolution, meaning the distance between the radioactive compound and the activated silver grain, depends on which radioisotope is used. Higher energy-isotopes, like \textsuperscript{125}I, may activate silver grains at a greater distance than lower energy radioisotopes, such as tritium\textsuperscript{184}. In \textbf{Paper II}, the brains were cryosectioned, and the brain capillaries were immunofluorescently stained. This allowed us to distinguish between the NTE signal within the brain capillaries and the signal in the brain parenchyma.

Thin-layer chromatography (TLC)

In \textbf{Paper II}, Thin-layer chromatography (TLC) was used to check if \textsuperscript{125}I was attached to the antibody constructs in the plasma and urine or if the \textsuperscript{125}I was detected in free form. As well as to check for the relative levels of \textsuperscript{125}I-labeled antibody constructs. A drop of plasma or urine was applied to a TLC plate and put in a solvent chamber. After the solvent was run for around 10 cm, the plate was dried, exposed to a phosphor imaging plate, and scanned with a phosphor imager. Large molecules, like \textsuperscript{125}I-labeled antibody constructs, stay at the application spot, while small molecules, such as free \textsuperscript{125}I, move with the solvent and separate.
Aims

One aim of this thesis was to develop a small-scale transfection protocol to optimize protein expression of antibody therapeutics (Paper I). The overall aim of the remaining studies (Paper II, III, and IV), was to investigate the binding properties of the BBB shuttle scFv8D3 and attempt to improve its performance through protein engineering.

Specific aims

I. Updating our TGE protocol for expressing antibody therapeutics and developing a small-scale version of said TGE protocol to enable testing conditions that may improve the production output (Paper I).

II. To investigate if HS binding affects the brain uptake of scFv8D3 and if adding additional HS binding sites can improve its brain uptake (Paper II).

III. To investigate if the strategy of fine-tuning the affinity of TfR-binding BBB shuttles to prolong blood half-life and enhance brain delivery is a viable strategy for scFc-scFv8D3 (Paper III).

IV. To investigate if fusing holoTf to our previously published partly bivalent antibody therapeutic RmAb158-scFv8D3 could increase its transcytosis (Paper IV).
Summary of investigations

Paper I

In Paper I, the aims were to detail the improvements to our previously published TGE protocol (part I) for the expression of antibody therapeutics and to develop a small-scale version of said TGE protocol (part II) to surmount the cost limitation of testing many culture conditions with the aim of improving protein production output.

In part I, we updated our highly cited TGE protocol\textsuperscript{28} by detailing the change to a much cheaper expression medium and a quicker and simpler harvesting procedure. Replacing the expression medium decreased its cost by approximately 90%. The harvesting procedure of the TGE protocol was shortened from 2.5 hours to 15 minutes by mixing the harvested cell media with refined diatomaceous earth, which is a mineral compound with properties that allow it to absorb and sequester cells and cell debris, after which the cell media can be quickly filtered without the need to exchange obstructed filters and without the previously needed 1-hour centrifugation step.

In part II, a small-scale version of the TGE protocol was developed and tested by evaluating the use of longevity molecules as potential protein expression enhancers. By quantifying the antibody yield by ELISA, we showed that the small-scale TGE had protein expression comparable to the TGE protocol, meaning that the nanomolar yields were in the same order of magnitude. In the small-scale TGE protocol, the consumption of materials was decreased by 83% by using 6-well plates for the expression, compared to transfections done with the smallest available shaking flasks for cell culture. However, we observed a large variation in protein expression both for the small-scale TGE and the TGE in shaking flasks, and none of the longevity molecules seemed to enhance the protein expression of the small-scale TGE in the conditions we tested. The small-scale TGE protocol did surmount the cost limitation of testing many cell culture conditions by markedly decreasing the cost per transfection. Still, due to the aforementioned considerable variation in protein expression, the small-scale TGE protocol can only detect large effects on protein expression, which limits its application. Our findings highlight the importance of reproducing results indicating increased TGE before concluding the success of any given culture condition optimization.
In Paper II, the aim was to investigate if HS binding affects the brain uptake of the BBB shuttle scFv8D3 and if adding additional HS binding sites can improve its brain uptake. The binding of proteins to the negatively charged HS chains requires positively charged HS binding sites. HS binding sites exist as linear peptide regions (HS binding motifs) or nonlinear higher-order HS binding clusters. To start with, a 3D model of a single chain fragment variable (scFv) of the anti-mouse TfR (mTfR) antibody 8D3 (scFv8D3) was generated by homology modeling to allow for searching the surface of scFv8D3 for HS binding sites present, as well as for regions suitable for introducing new sites. One potential HS binding cluster and one potential HS binding motif were identified in scFv8D3. No regions suitable for creating new HS binding motifs were found. However, two regions were found where a positively charged amino acid could be introduced at the correct distance, creating two new potential HS binding clusters. We then designed two scFv8D3 mutants: HS(-)scFv8D3 with reduced HS binding sites and HS(+)scFv8D3 with additional HS binding sites.

The HS mutants were tested in vitro with ELISA and Thermal shift assays, confirming that they retained their mTfR binding capacity and structural stability. Then, their HS binding capacity was assessed by heparin liquid chromatography, which showed that scFv8D3 and HS(-)scFv8D3 could not bind to the column, while HS(+)scFv8D3 could bind to it. Finally, the brain uptake, biodistribution, plasma stability, and renal elimination of $^{125}$I labeled antibody constructs were investigated ex vivo/in vivo after intravenous injection in WT mice. The ex vivo brain uptake results demonstrated that the brain uptake of scFv8D3 is not influenced by the HS binding sites identified and that the additional HS binding sites we added did not improve its brain uptake. The brain uptake results were confirmed with capillary depletion and microautoradiography. In contrast to the brain uptake findings, the addition of additional HS binding sites markedly decreased the urine concentration of the HS(+)scFv8D3 mutant, which was observed when analyzing the urine with TLC.

Based on our results, we consider it plausible that HS binding is not an important property for the brain uptake of the scFv8D3 brain shuttle. This finding contributes to the effort of evaluating protein engineering strategies to enhance brain delivery of antibody therapeutics. Our results demonstrate that the design and approach that we used in the study can be employed to add HS binding sites to the surface of antibody constructs and that doing so can decrease the renal elimination of small (≤ 50 kDa) antibody constructs, which might be of use if one wants to target the tubular endothelium of the kidney.
Paper III

In Paper III, the aim was to investigate if the strategy of fine-tuning the affinity of TfR-binding BBB shuttles to increase brain delivery and half-life is a viable strategy for the monovalent scFv-scFv8D3 BBB shuttle. Fine-tuning the affinity of high affinity TfR binders by moderately decreasing their affinity can be done by targeting amino acids with point mutations, and this has previously been reported to be effective for bivalent (binding with two binding domains to its target) 8D3 constructs. To identify amino acids likely to influence TfR affinity, the amino acids within scFv8D3-s VHCDR3 and the amino acids within its epitope on mTfR were investigated by theoretical functional group analysis and in silico protein-protein docking analysis. To enable the protein-protein docking, a 3D model of a shortened mTfR peptide retaining the mTfR epitope of scFv8D3 was generated with AlphaFold and used with the previously generated scFv8D3 homology model. The identified amino acids within scFv8D3-s VHCDR3 were replaced by single point mutations, creating five affinity mutants of scFv8D3.

Mutating the identified amino acids decreased the mTfR affinity of the scFv8D3 mutants by 7- to 23-fold for four of the five affinity mutants, with one having almost abolished binding, demonstrated by mTfR ELISA. Using our previously published in vitro BBB model, the In-Cell Trans assay, the affinity mutants were screened to assess their transcytosis capacity. The mutant with almost abolished TfR-binding was shown to be unable to cross the BBB model and was, therefore, not included in the subsequent animal study. The selected affinity mutants were 125I labeled and intravenously injected into WT mice, and their half-life in blood was determined over the course of a five-day experiment, after which the animals were terminated. Subsequently, the biodistribution of the 125I labeled affinity mutants was investigated ex vivo. The affinity mutants were shown to have prolonged blood half-life (1.3-fold increase to 2.6-fold increase), and our preliminary results indicate increased brain concentrations 120- hours post-injection (no increase to 1.7-fold increase).

The increase in blood half-life and brain concentration were observed to correlate non-linearly with decreased affinity. For both properties, moderately reducing the affinity had the best effect, which is in accordance with previous findings reported for bivalent 8D3 affinity mutants. By using our In-Cell Trans assay, we were able to distinguish the effect of lowering TfR affinity on transcytosis from the effect on brain concentration as a result of prolonged blood half-life, where the latter was indicated to be the factor that ultimately increases brain concentration. Monovalent BBB shuttles are seemingly superior for BBB transcytosis at therapeutically relevant doses and, to the best of our knowledge, this is the first study showing that the strategy of
fine-tuning the affinity of TfR antibodies is not only beneficial for delivering therapeutic doses of bivalent 8D3 constructs but for monovalent 8D3 constructs as well. Our findings corroborate the growing amount of data supporting the strategy, and our approach may be relevant for others attempting to optimize brain delivery.
In Paper IV, the aim was to investigate if fusing holoTf to our previously published partly bivalent BBB shuttle RmAb158-scFv8D3 could increase its transcytosis. The iron transport from blood circulation to the brain is mediated by TfR’s natural ligand Tf, specifically the iron-loaded form holoTf. Non-competitive and non-bivalent TfR binding has previously been shown to be beneficial for anti-TfR BBB shuttles, such as 8D3. In this paper, we redesigned RmAb158-scFv8D3 that binds mouse TfR partly bivalently due to its design; the antibody’s TfR binding arms consist of scFv8D3 fused to the C-terminal of the antibodies light chain via a short flexible linker that sterically hinders bivalent TfR-binding. The new design was made by fusing recombinant mouse holoTf to the end of each of the antibody's TfR binding arms via the aforementioned short flexible linker, creating the RmAb158-scFv8D3-holoTf antibody.

To test the functionality of the antibody, TfR ELISA was performed, showing that RmAb158-sFv8D3-holoTf binds TfR much stronger than the original design, nearly as strong as a bivalent 8D3 IgG antibody. This result indicated that the fusion of holoTf shifted the binding properties of the antibody from partly bivalent to fully bivalent. To test the transcytosis efficiency of the antibody, our In-Cell Trans assay was used. Unfortunately, the transcytosis of RmAb158-sFv8D3-holoTf was observed to be significantly diminished compared to the original design, indicating that the potential transcytosis-promoting effect of the fused holoTf was absent and/or counteracted by the antibodies TfR-binding properties.

The results of this present paper corroborate the growing amount of evidence suggesting that bivalent TfR binding decreases transcytosis, which might be due to cross-linking of TfR receptors on the cell surface and/or by preventing adequate dissociation from TfR. Further, we can speculate that holoTf-fusion as a strategy may still prove to be useful for monovalent antibody designs.
Conclusions and future perspectives

In this thesis, we have studied strategies for improving the brain delivery of antibody-therapeutics into the brain by investigation and protein engineering of TfR-binding BBB antibody shuttles, and a secondary focus was on improving their expression, as producing sufficient protein for experimental work can often be a limitation when developing antibody therapeutics.

Improving the expression of antibody therapeutics

Paper I of this thesis attempted to contribute to the effort of improving the expression of antibody therapeutics by sharing our improved TGE protocol (part I) and by developing a small-scale version of the protocol to surmount the cost-limitation of testing many culture conditions to improve the protein production output (part II). Since most research groups do not publish the updates they make to their protocols, we believe that though our improvements may not necessarily be unique, sharing them with the scientific community can help further the advancement of the field by decreasing the cost of developing new antibody therapeutics. Similarly, in part II, the small-scale TGE protocol effectively reduced the cost of testing many culture conditions. However, the large variation of protein expression observed in both protocols raises an important issue with optimizing the TGE of antibody therapeutics. The variation may produce false-negative results as enhancements of protein expression can be occluded, and inversely, results indicating enhancement may be due to the inherent variation. This highlights the importance of reproducing one's results before concluding the success or failure of optimizing culture conditions for TGE. The cause of this problematic variation of protein expression may be multifactorial as all DNA delivery methods used in TGE exhibit variability in transfection efficiency\(^{186}\) and confer some measure of cell toxicity\(^{20}\), both of which may influence protein expression. Future studies should, therefore, focus on investigating the cause of the variability in protein expression and provide insights into ways to decrease it, which would be useful in itself and allow future studies into the optimization of protein expression.
Improving the design of antibody BBB shuttles

Paper II, Paper III, and Paper IV of this thesis contribute to knowledge about the properties and design of TfR-BBB shuttles that affect their capacity to reach the brain, which is paramount for enabling the use of antibody therapeutics to treat brain disorders. In Paper II, we concluded that HS binding is likely not a factor that influences the transcytosis of the BBB shuttle scFv8D3. However, it is possible that the HS-binding sites we identified on scFv8D3 and added to scFv8D3 do not bind to the HS present at the BBB. As previously mentioned, HS chains are very diverse in their structure, and the HSPGs present at the surface of different tissues also vary. Therefore, further studies are required to evaluate if adding HS binding sites to BBB shuttles can improve their transcytosis. Basic research into what specific HS targets are available at the BBB and the role (if any) that HSPGs at the BBB play in regulating endocytosis would greatly facilitate further research evaluating the strategy. Finally, the results of Paper II indicate that our approach of introducing sequence-distant point mutations to create higher-order HS-binding clusters was successful. This approach might be useful to others wanting to engineer HS-binding sites into BBB shuttles.

In Paper III, we investigated if lowering (fine-tuning) the affinity of our monovalent scFc-scFv8D3 BBB shuttle could prolong its blood half-life and lead to higher brain concentrations when administering a therapeutic dose. As mentioned previously, both affinity and binding valency are properties of TfR binders that are believed to influence the effectiveness of BBB shuttles, where monovalent binding is seemingly superior to bivalent binding.

Others have previously shown that fine-tuning the affinity of other TfR binders (both bivalent and monovalent constructs) can improve their performance. However, to our knowledge, our study was the first to investigate if the strategy is beneficial for delivering therapeutic doses of monovalent 8D3 constructs and to distinguish the effect on transcytosis from the effect on brain delivery. Lowering the affinity of antibodies can be done by introducing point mutations within their CDR regions to weaken epitope interaction. Our approach of functional-group analysis, combined with and corroborated by in silico protein docking, proved useful in determining which amino acids mutate and substantially decreased the time and effort needed compared to the commonly used alanine-scanning methodology. The mutations we introduced successfully created affinity mutants of scFv8D3 that when tested, exhibited lowered TfR affinity, longer blood half-life, and higher brain concentration 120 hours post-injection, thereby strengthening the case for applying the fine-tuning strategy for monovalent 8D3 constructs. By utilizing our In-Cell Trans assay, we were able to differentiate the impact of decreasing TfR affinity on transcytosis from the effect on brain concentration caused by extended blood
half-life, and our results indicate that it is the latter factor that ultimately increases brain concentration of the monovalent 8D3 affinity mutants. Therefore, we may speculate that increased transcytosis might not be the factor that improves brain delivery of some monovalent TfR-binders with fine-tuned TfR affinity. As previously mentioned, Bien-Ly et al. observed that therapeutic dosage of a high affinity monovalent bispecific anti-TfR antibody (Anti-TfR\textsuperscript{A}/BACE1) caused degradation of TfR and decreased in vivo transcytosis\textsuperscript{142}. In comparison, the medium affinity mutant (Anti-TfR\textsuperscript{D}/BACE1) created by Couch et al.\textsuperscript{144} caused less TfR degradation. Surprisingly, it was reported to exhibit higher brain concentration even at the early time point of 24 hours\textsuperscript{142}, whereas in Couch's study,\textsuperscript{144} the same Anti-TfR\textsuperscript{D}/BACE1 antibody did not outperform Anti-TfR\textsuperscript{A}/BACE1 at 24 hours or 48 hours but only after 4 days post-injection, which was attributed to markedly prolonged plasma half-life of Anti-TfR\textsuperscript{D}/BACE. Taken together, these results suggest that fine-tuning the affinity of monovalent TfR-binders might be beneficial for brain delivery at therapeutic doses both due to prolonged half-life and increased transcytosis.

In addition to brain delivery of antibody therapeutics, targeting nanomedicine to the brain by conjugation of anti-TfR antibodies has been explored. In a study by Johnsen et al.\textsuperscript{178}, the researcher compared the brain delivery of gold-nanoparticles using the abovementioned high affinity monovalent Anti-TfR\textsuperscript{A}/BACE1 antibody, the original bivalent high affinity antibody (Anti-TfR\textsuperscript{A})\textsuperscript{41}, and the bivalent medium affinity mutant (Anti-TfR\textsuperscript{D})\textsuperscript{41}. Of note, the affinity of the monovalent TfR\textsuperscript{A}/BACE1 and the bivalent Anti-TfR\textsuperscript{A} was reported to be similar.\textsuperscript{178} The researchers found that Anti-TfR\textsuperscript{D} outperformed Anti-TfR\textsuperscript{A}, which exhibited intermediate and low increases in brain delivery, respectively. However, the use of the monovalent Anti-TfR\textsuperscript{A}/BACE1 resulted in a three-fold higher brain concentration compared to Anti-TfR\textsuperscript{D}. These results indicate that monovalency has a bigger impact than fine-tuned TfR affinity for the brain delivery of nanoparticles. However, it must be noted that the total ligand avidity of nanoparticles is also an important factor that affects their brain delivery,\textsuperscript{189,190} and due to the fact that several anti-TfR binders are present on the surface of the nanoparticles, it is possible that the effect of bivalent binding becomes enhanced.

An interesting future study would be to compare the in vitro transcytosis (InCell Trans assay), blood half-life, and brain delivery performance of a bivalent 8D3 IgG-affinity mutant construct paired up with a monovalent asymmetric 8D3 IgG construct having the same affinity. Such a study might provide valuable insight into whether the benefits of a monovalent binder are due to valency or decreased affinity (loss of avidity) and whether the benefits of lowered affinity are due to increased transcytosis and/or prolonged half-life. In addition, as previously mentioned, there are currently antibody therapeutics
with both monovalent and bivalent BBB shuttles in clinical trials targeting the same brain disorder, and the outcome of these trials will hopefully increase the knowledge about which format is more effective at delivering antibody therapeutics to the brains of humans.

Another factor that might influence the transcytosis of BBB shuttles is holoTf binding to TfR. In Paper IV, we investigated if fusing holoTf to our previously published partly bivalent BBB shuttle RmAb158-scFv8D3 could increase its transcytosis. However, the new RmAb158-scFv8D3-holoTf design had a much stronger TfR affinity, similar to that of bivalent 8D3 IgG, indicating a shift from partly bivalent binding to fully bivalent. Perhaps not surprisingly, we observed a significant decrease in the efficiency of transcytosis for RmAb158-scFv8D3-holoTf compared to the original design. This suggests that the fused holoTf did not have the potential to promote transcytosis or that the TfR-binding properties of the antibody counteracted it. The findings of this study support the growing evidence indicating that bivalent TfR binding reduces transcytosis, potentially due to cross-linking of TfR receptors on the cell surface\textsuperscript{140,185}, and/or by preventing adequate dissociation from TfR\textsuperscript{142–144}. However, studies by other members of our research group have recently shown that supplementation with holoTf can enhance the transcytosis of TfR-binding antibodies, regardless of their TfR binding valency. This effect was observed both in our in vitro BBB model and in vivo (unpublished results). Based on these findings and the results of Paper IV, we may speculate that holoTf fusion as a protein engineering strategy may still be beneficial for monovalent BBB shuttles, which could be investigated in future studies.
Antibodies, also known as immunoglobulins, play a crucial role in our immune system by protecting us from harmful pathogens like viruses and bacteria that enter our body. Antibodies have evolved to bind their targets with remarkable specificity and strength (affinity), which make them ideal candidates for creating a class of biological drugs called antibody therapeutics. Antibody therapeutics refer to protein molecules that contain at least one binding site obtained from an antibody gene, and they are now used as potent drugs that are customizable by protein engineering. The customizability of antibody therapeutics is a great advantage, as it is possible to adapt them to the specific needs of the drug, such as binding multiple targets. The success of antibody therapeutics in treating various diseases is owed to this customizability, their unique binding specificity, affinity, and generally causing fewer side effects compared to other drugs. The field of antibody therapeutics has experienced significant growth, and currently, there are nearly 1200 antibody therapeutics in clinical trials. However, unfortunately, only a few of them target brain diseases, as antibody therapeutics cannot passively travel from the blood circulation into the brain.

This is because of a protective barrier called the blood-brain barrier (BBB), which prevents large molecules from reaching the brain, and antibody-therapeutics are much larger than traditional non-biological small-molecule drugs (up to a few thousand-fold larger). Brain delivery of antibody therapeutics can be done by binding receptors at surface of the BBB that naturally transport proteins into the brain, such as the Transferrin receptor (TfR). This strategy is referred to as the molecular Trojan horse, and the parts of brain-penetrating antibody therapeutics that bind to the receptor are called BBB shuttles.

This thesis has mainly studied properties of TfR-binding BBB shuttles that are important for their performance, as well strategies for their improvement (Papers II, III, and IV) and how to enhance the protein expression of antibody therapeutics to improve production output (Paper I).

When developing new antibody therapeutics, it is desirable to produce them quickly. This can be achieved through a method called transient gene expression (TGE). Although TGE has been shown to be a good method for protein
expression by our research group and others, there is always room for improvement. This could help save costs and help achieve sufficient protein yields for experimental work. In Paper I, we shared our updated TGE protocol and developed a small-scale version to surmount the cost-limitation of testing multiple conditions. However, both protocols showed significant variation in protein expression, which is not desirable due to decreased reliability and also hinders optimization studies as the results could be obscured by the variation. This problematic variation prompts future studies investigating its cause(s).

In paper II, we investigated if heparan sulfate (HS) binding could improve brain delivery of the BBB shuttle scFv8D3. HS is a type of sugar molecule that exists as long negatively charged chains. These chains vary exceedingly in length and sulphation patterns and are attached to a group of proteins called HS proteoglycans (HSPGs). HSPGs are present at the surface of the BBB and have been shown to be involved in the transport of many types of large molecules into cells. Our findings suggest that the BBB shuttle scFv8D3 does not rely on the HS binding sites that we identified. Additionally, our attempts to improve the shuttle's performance by adding new sites were unsuccessful. However, given the intricacies of the HS target, more research is necessary to gain a deeper understanding and enable future studies to determine the viability of the strategy.

It has been demonstrated earlier that decreasing the TfR affinity (binding strength) of BBB shuttles can enhance the delivery of therapeutic doses of 8D3 antibodies that bind with two domains (bivalent binding). Binding TfR with too strong affinity is believed to cause shuttles to not properly dissociate from the receptor and therefore get degraded within the cells of the BBB instead of transported through (transcytosis). Bivalent binding of TfR is similarly believed to decrease transcytosis as binding with two domains results in much stronger affinity. Alternatively, the decreased transcytosis might be due to bivalent binders’ ability to cross-link TfR receptors which is also believed to lead to degradation. Another factor important for brain delivery is the duration of time for which TfR-binding shuttles remain in the bloodstream, known as blood half-life. Generally, a longer half-life leads to greater brain exposure and therefore enhanced brain delivery. The blood half-life of TfR-binding shuttles is inversely proportional to their affinity for the TfR receptor. Therefore, shuttles with moderate TfR affinity tend to have a longer blood half-life and result in more effective drug delivery to the brain. In Paper III, we employed the strategy of lowering TfR affinity to the monovalent scFc-scFv8D3 antibody. We created affinity mutants of scFc-scFv8D3 that exhibited lower TfR affinity, longer blood half-life, and higher brain concentration 120 hours after tail vein injections into mice, thereby strengthening the case for applying the strategy for monovalent 8D3 constructs. Our In-Cell Trans assay helped us distinguish between the impact of decreasing TfR affinity on transcytosis
and the effect on brain concentration caused by extended blood half-life. Our findings suggest that it is the latter factor that ultimately increases brain concentration. Hence, we can speculate that improved brain delivery of monovalent TfR-binders with fine-tuned TfR affinity is not only due to increased transcytosis.

The TfR receptor is responsible for transporting iron from blood circulation into the brain, which it does via the iron-transporting protein transferrin. When transferrin carries iron, it is referred to as holo-transferrin (holoTf). In paper IV, we investigated if the fusion of holoTf to a brain-penetrating antibody therapeutic antibody could improve its transcytosis. The original design comprised a therapeutic IgG antibody with two TfR binding arms consisting of scFv8D3 attached via a short flexible linker, designed to hinder bivalent TfR-binding, making it only partly bivalent. To the end of each TfR binding arm, we attached holoTf via the same short linker. Based on our findings, it appears that the new design's TfR binding has shifted from partly to fully bivalent, leading to a significant reduction in in vitro transcytosis. This means that the potential transcytosis-promoting effect of the fused holoTf was either absent or counteracted by the design's bivalent high affinity binding. However, the strategy may still prove to be useful for monovalent TfR binders, which could be investigated in a future study.

In conclusion, the findings of this thesis corroborate that monovalent and low-to-moderate affinity binding are likely beneficial properties for TfR-mediated brain delivery of therapeutic doses of antibody therapeutics. Regarding the strategies for improving brain delivery explored in the thesis our results support fine-tuning TfR affinity. However, whether it is possible to enhance brain delivery by added HS-binding sites or by holoTf-fusion requires further study. Taken together, the work performed in this thesis contributes to the knowledge about the properties and design of TfR-BBB shuttles that affect their capacity to reach the brain.


Denna avhandling har främst studerat egenskaper hos TfR-bindande BBB-transportörer som är viktiga för deras prestanda och strategier för att förbättra prestandan (Paper II, III och IV), samt hur man kan förbättra proteinuttrycket av antikroppsämne för att öka deras produktion (Paper I).

I artikel II undersökte vi om bindning till heparansulfat (HS) kunde förbättra leveransen av BBB-transportören scFv8D3 till hjärnan. HS är en typ av sockermolekyl som existerar i form av långa negativt laddade kedjor. Dessa kedjor varierar kraftigt i längd och sulfateringsmönster, samt är bundna till en grupp proteiner som kallas HS-proteoglykaner (HSPGs). HSPGs finns på ytan av BBB och de har visat sig vara inblandade i transporten av många typer av stora molekyler in i celler. Våra forskningsresultat tyder på att BBB-transportören scFv8D3 inte är beroende av de HS-bindningsställen som vi identifierade. Därtill misslyckades våra försök att förbättra transportörens prestanda genom att lägga till nya bindningsställen. På grund av hur varierat och intrekat HS är behövs en djupare förståelse kring dess egenskaper vid BBB innan man kan utforma nya studier för att bedöma ifall strategin är användbar.

Det har tidigare visats att en minskning av TfR-affiniteten hos BBB-transportörer kan förbättra transporten av terapeutiska doser av 8D3-antikroppar som binder med två domäner (bivalent binding). Bindning av TfR med för stark affinitet tros leda till att transportörerna inte dissociierar i tillräcklig utsträckning från receptorn och därför bryts ned inuti BBB istället för att transportereras igenom dess celler (transcytosis). Bivalente binding av TfR tros på liknande sätt minska transcytosen eftersom bindning med två domäner resulterar i mycket starkare affinitet. Alternativt kan den minskade transcytosen bero på bivalenta bindares förmåga att korslänka TfR-receptorer, vilket också tros leda till nedbrytning. En annan faktor som påverkar hur mycket av antikroppsläkemedlet som når hjärnan är hur länge TfR-bindande transportörer finns kvar i blodomloppet, den s.k. halveringstiden i blodet. Generellt leder en längre halveringstid till bättre exponering mot hjärnan över tid och därmed näs högre koncentrationer hjärnan. Halveringstiden i blodet för TfR-bindande transportörer är omvänt proportionell mot deras affinitet till TfR-receptorn. Därför tenderar
transportörer med måttlig TfR-affinitet att ha en längre halveringstid i blodet och därmed förbättrad läkemedelstillförseln till hjärnan.


Sammanfattningsvis bekräftar resultaten i denna avhandling att monovalent bindning med låg till måttlig affinitet sannolikt är fördelaktiga egenskaper för att leverera terapeutiska doser av TfR-bindande antikroppsläkemedel till hjärnan. Förlängd halveringstid i blodet verkar vara en viktig orsak till fördelarna med att sänka affiniteten hos monovalenta TfR-transportörer. Huruvida det är möjligt att förbättra leveransen till hjärnan genom att lägga till HS-bindningsställen eller genom att länka holoTf till antikroppsläkemedel kräver dock ytterligare studier. Sammantaget bidrar arbetet i denna avhandling till kunskapen om de egenskaper och designer av TfR-transportörer som påverkar deras förmåga att tillföra antikroppsläkemedel till hjärnan.
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Schematic images were created by the author in part using BioRender. Sections of the text written in this thesis was polished with help of the tool Grammerly.
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References


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