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Targeting pathological alpha-synuclein

Protein engineering towards improved antibody-based therapeutics and their delivery to the brain

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Abstract
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The aggregation of alpha-synuclein (αSyn) into oligomers and fibrils is central to the disease progression of Parkinson's disease and related pathologies, where αSyn aggregates spread between neurons and cause neurodegeneration. To this date, there is no treatment available that could interfere with the aggregation of αSyn to potentially stop the disease progression. Among the major limitations in the development of therapeutics against αSyn aggregation are the low extracellular concentration of αSyn, the low selectivity of therapeutics for the pathologically relevant αSyn species, and the lacking detailed knowledge about the actual pathological αSyn species.

In this thesis, different engineered antibodies and αSyn mutants were investigated with the aim to identify better strategies of antibody-based treatment of αSyn aggregation.

In Paper I, we engineered multivalent antibodies based on the αSyn aggregate-specific antibody SynO2 to enhance the antibody's binding strength to a wide range of soluble αSyn aggregates. We could show that the higher valency increased the binding strength to αSyn aggregates up to 20-fold.

In Paper II, we aimed to improve the design of the antibody RmAb158-scFv8D3 to enhance its TfR-mediated brain uptake. By drastically reducing the linker length between the therapeutic antibody and its TfR-targeting scFv8D3, we showed a two-fold enhanced transcytosis across an in vitro BBB model.

In Paper III, we fused a negatively charged peptide to the αSyn aggregate-specific antibodies SynO2 and 9E4 to test whether those fusion antibodies had the potential to bind with higher avidity to αSyn aggregates. Our results showed lower binding strengths compared with the parental antibodies.

In Paper IV, we designed αSyn mutants with a stabilized beta-hairpin conformation to produce stable, small αSyn oligomers closely resembling native, pathological αSyn oligomers. We showed that two of the mutants formed exclusively pentameric and hexameric oligomers under conditions that promoted fibrillation of wild-type αSyn.

In conclusion, this thesis shows that increasing the valency of an antibody is a possible strategy to enhance its binding strength to αSyn aggregates. However, to effectively target pathologically relevant αSyn species, a more selective targeting approach may be required, possibly through a conformational epitope exclusive to αSyn oligomers.

Keywords: Parkinson's disease (PD), alpha-synuclein (αSyn), beta-hairpin, protein drugs, multivalent antibodies, blood-brain barrier (BBB), transferrin receptor (TfR)

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To my family, friends, and everyone who has supported me

List of Papers

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

I. **Petersen I.**, Ali M. I., Petrovic A., Ytterberg A. J., Staxäng K., Hodik M., Rofo F., Bondza S., Hultqvist G. (2023) Multivalent design of the monoclonal SynO2 antibody improves binding strength to soluble α-Synuclein aggregates. *mAbs*, 15(1):2256668.

II. **Petersen I.**, Morrison J. I., Petrovic A., Babic N., Metzendorf N., Godec A., de la Rosa A., Rofo F., Bondza S., Buijs J., Ranjbarian F., Hofer A., Sehlin D., Hultqvist G. (2024) A shorter linker in the bispecific antibody RmAb158-scFv8D3 improves TfR-mediated Blood-Brain Barrier transcytosis *in vitro*. *Submitted manuscript.*

III. **Petersen I.**, Godec A., Ranjbarian F., Hofer A., Mirabello C., Hultqvist G. (2024) A charged tail on anti-α-Synuclein antibodies does not enhance their affinity to α-Synuclein fibrils. *Accepted manuscript in PlosONE.*

IV. **Petersen I.**, Staxäng K., Hodik M., Leroy P., Hultqvist G. (2024) A stable beta-hairpin conformation stabilizes alpha-synuclein as small oligomers. *Manuscript.*

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Abbreviations

αSyn	Alpha-synuclein
^{125}I	Iodine-125
AF2	AlphaFold2
Aβ	Amyloid-beta
BBB	Blood-brain barrier
CD	Circular dichroism
CDR	complementary-determining region
CPP	Cell-penetrating peptide
DLS	Differential scanning fluorimetry
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcRn	neonatal Fc receptor
FcγR	Fc gamma receptor
HNE	4-hydroxy-2-nonenal
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
Ig	Immunglobulin
k_a	Association rate
k_d	Dissociation rate
K_D	Affinity
kDa	Kilodalton
LB	Lewy body
mTfR	murine TfR
NAC	Non-amyloid-beta component
PD	Parkinson's disease
PFF	Preformed fibrils
scFc	Single-chain fragment crystallizable
scFv	Single-chain fragment variable
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy
Tf	Transferrin
TfR	Transferrin receptor

ThT	Thioflavin T
TMB	Tetramethylbenzidine
V_H	Variable heavy chain
V_L	Variable light chain

Introduction

Protein-based biological drugs

Biological drugs or biopharmaceuticals, opposed to the most common chemical drugs, are active drug substances that are produced by living cells, which can be nucleic acids, carbohydrates, peptides or proteins, or even viruses or cells. With human insulin being the first recombinantly produced protein-based drug in 1978, recombinant protein-based drugs represent the majority of biological drugs on the market today [1].

Of the wide spectrum of functions of protein drugs, the most common ones are to replace a deficient protein, to enhance an endogenous pathway or to interfere with a pathway, pathogen or pathogenic molecule. In contrast to chemical drugs, protein-based drugs are very large, with complex structures, which give them high affinity and specificity to their target, but also causes a number of challenges for the production and handling of protein-based drugs. The structure of protein-based drugs is often not fully characterized and the stability can vary strongly with small changes in the production, downstream processing and storage conditions and thus affect activity and immunogenicity of the protein drug [2]. Additionally, the size of protein-based drugs can be up to several hundred kilodalton (kDa), as in the case of antibodies, which limits their distribution in the body since molecules of that size cannot cross cellular membranes on their own. Nevertheless, monoclonal antibodies are the most rapidly growing type of biological drugs today with over 100 therapeutic antibodies approved by the food and drug administration in the US in 2021 [3].

Antibodies

Antibodies are protein complexes of two identical light and two identical heavy chains that are stabilized through interchain disulfide bonds (Fig. 1 a). Traditionally, the two N-terminal antibody “arms” are referred to as the fragment antigen-binding (Fab) regions and the C-terminal “stem” as the fragment crystallizable (Fc) region (Fig. 1 b). The N-terminal half of a Fab is referred to as the variable domain, and constitute the actual antigen binding site. A variable domain is characterized by three highly variable amino acid stretches, referred to as complementarity-determining regions (CDR), which are interrupted by more conserved framework sequences. The high variability and specificity of antibodies is naturally the result of extensive alternative splicing

of antibody encoding mRNA transcripts. Additionally, the ability of an antibody to bind with its two arms to two antigens on a multimeric or membrane-bound target simultaneously can increase its total affinity by multiple times which is called avidity-effect and is owed to the decreased chances of diffusion of the antibody. The C-terminal half of each Fab together with the Fc part constitute the constant region, which is identical for all antibodies within one antibody subclass. There are five isotypes of antibodies, IgA, IgD, IgE, IgG and IgM, and additional subclasses within. Besides the conserved sequence of the constant region, each antibody subclass is also characterized by specific glycosylation patterns on the Fc region, which have important effects on the antibodies' effector functions and half-life [4,5].

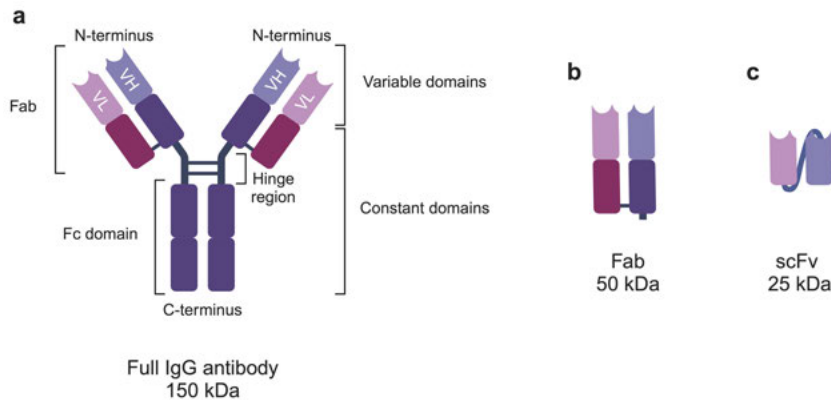

Figure 1. Schematic illustration of the structure of an IgG antibody and antibody fragments. (a) Two identical heavy chains (blue) and two identical light chains (purple), which are linked together by disulfide bonds, form an IgG antibody. The hinge region connects the Fab (fragment antigen-binding) with the Fc (fragment crystallizable) domain. The N-terminal domains constitute the variable domains (VH: variable heavy; VL: variable light). The C-terminal domains constitute the constant domains. (b) A Fab consists of the variable light and variable heavy domains and the adjacent constant light and heavy domains above the hinge region. (c) A single-chain fragment variable (scFv) consists of the variable light and variable heavy domains connected through an amino acid linker.

Antibodies are part of the adaptive immune system where they are produced by B-cells as response to the recognition of a foreign antigen. The possible functions of an antibody comprise target-neutralization upon binding of the antibody's antigen binding site to the antigen, which can result in blocking an active site of the target molecule or cell. The antigen-antibody complexes can subsequently be eliminated through phagocytosis, induced by the interaction between the antibody's Fc part and Fc gamma receptors (FcγRs) on specific immune cells such as natural killer cells, macrophages, monocytes and dendritic cells. The affinity of different FcγRs varies between the different antibody isotypes and subclasses and hence determines the strength of effector

functions that an antibody can induce [6]. Additionally, IgG antibodies can bind to the neonatal Fc receptor (FcRn), which enables IgG recycling upon endocytosis into vascular endothelial cells, protecting IgG antibodies from lysosomal degradation and thus explains their long serum half-lives [7]. Thanks to these properties, IgG antibodies have become a key player in the field of therapeutic proteins. Additionally, antibodies are an essential tool in molecular biological in vitro assays, such as the enzyme-linked immunosorbent assay (ELISA) and western blot, to determine the presence and/or concentration of specific proteins in a sample or to study protein-protein interactions.

Therapeutic antibodies

Since the first therapeutic antibody was approved in 1985 [8], a lot has changed in the field of generating and engineering therapeutic antibodies. Monoclonal antibodies, describing a group of identical antibodies that bind to only one specific epitope on the antigen, are preferred for therapeutic applications opposed to polyclonal antibodies, which consist of a heterogeneous mix of antibodies binding to different epitopes on the respective antigen.

The traditional way of generating monoclonal antibodies against a specific antigen has been the hybridoma technique [9], which requires the immunization of an animal with the desired antigen, a subsequent fusion of extracted B-cells with myeloma cells and a selection of hybridoma clones producing antibodies with the desired properties. One major problem with antibodies obtained from classical hybridoma culture is the immunogenic response against the exogenous protein when injected into humans, resulting in the production of anti-drug antibodies and a rapid elimination of the therapeutic antibody [10]. The development of transgenic mice for the immunization step allowed the production of fully human antibodies and solved at least this problem of species-dependent immunogenicity. Alternatively, the technology of antibody phage display has become a popular way of generating antibodies, as it offers high through-put screening for favourable antibody characteristics and it does not require experimental animals as host organisms [11]. Today, antibodies are not only generated "de-novo" as by the methods mentioned above, but based on known antibody sequences, antibodies can be expressed in mammalian cell cultures. By recombinant antibody expression, one can not only make modifications of antibody domains such as exchanging the constant chain to reduce immunogenicity as well as single amino acid changes, but it also allows to create new antibody formats such as single-chain fragment variable (scFv) (Fig. 1 c) or whole antibody-fusion proteins (Fig. 2), where new domains are added to equip the antibody with additional functions.

Antibody fragments

Fabs (Fig. 1 b) have traditionally been generated by proteolytic cleavage of full antibodies *in vitro* using the protease papain, which cleaves the antibody in the hinge region, but Fabs can also be produced recombinantly [12,13]. ScFvs can only be produced recombinantly as they consist of respectively one variable heavy (V_H) and one variable light chain (V_L) which are connected through a flexible, internal amino acid linker of typically 12-20 amino acids in length (Fig 1 c). It is said, that scFv retain the full affinity and specificity for the respective antigen as the original antibody, however, structure and binding characteristics of scFv strongly depend on the individual variable domain sequences, as well as on the internal linker design and the chain orientation being V_H-V_L or V_L-V_H [14,15].

The lower structural complexity of scFvs and Fabs, compared to full antibodies, allows to produce them in bacterial expression systems, which makes them a cheaper alternative. Due to their small size, both scFvs and Fab can also be of advantage in cancer therapies offering a better tumor penetration compared to full antibodies [16]. However, the lack of an Fc region in scFvs and Fabs causes a strong reduction of their serum half-live and those antibody fragments cannot induce immune cell effector functions. Conversely, Fc chains can be used in fusion proteins to prolong their serum half-life and to achieve effector functions in proteins other than antibodies [17,18].

Bispecific antibodies

The most common examples of antibody-fusion proteins are bispecific antibodies where antibodies, scFv or Fab are fused together recombinantly to achieve simultaneous or successive binding to two different targets. Simultaneous binding of two targets can be used to for example recruit immune cells into tumors. Bispecificity can also increase the antibody's potency by binding for example two independent, tumor-associated antigens and thus reducing the risk for drug resistance to arise [6]. Bispecific antibodies also enable the delivery of a therapeutic antibody across biological barriers when one of the domains is targeting a receptor on that barrier, which in turn can induce transcytosis upon antibody binding.

Multivalent antibodies

Antibody engineering is also used to create antibodies with a higher valency, that is a higher number of identical binding sites, to achieve stronger effects by binding to the target. With increasing the number of identical antigen binding sites, an antibody's potency in neutralizing a soluble target increases as it can bind more antigens at the same time. Is the target a multimer with multiple epitopes on one molecule or a cell surface-bound target, the antibody's

binding strength even potentiates with increasing valency as the combined affinity of all binding sites results in avidity binding, which decreases the chances for the antibody to dissociate from the target. IgG antibodies are naturally bivalent, which potentially makes them bind targets by avidity in contrast to scFv or Fabs, which are by definition monovalent. However, various ways to produce recombinant multivalent scFv or Fab constructs have been described, such as multimerization through the fusion of scFv with multimerization domains [19,20] (Fig. 2 a) or cross-linking of Fabs [21]. Multimerization of scFv is also possible by simply decreasing the length of the internal linker (Fig. 2 b). Linker lengths of >12 amino acids are typically long enough for the V_L and V_H of one scFv to pair with each other in a native way. Shorter linkers sterically hinder the internal pairing of V_L and V_H and instead V_L and V_H pair between different molecules forming typically dimers, trimer or tetramers, depending on the linker length and the respective structure of the individual V_L and V_H chains [22,23]. Beside Fab and scFv multimerization, interest has also been on increasing the valency of full IgG antibodies to enhance the antibody's neutralization potency [24] as well as to increase the avidity to small oligomeric targets [25] (Fig. 2 c).

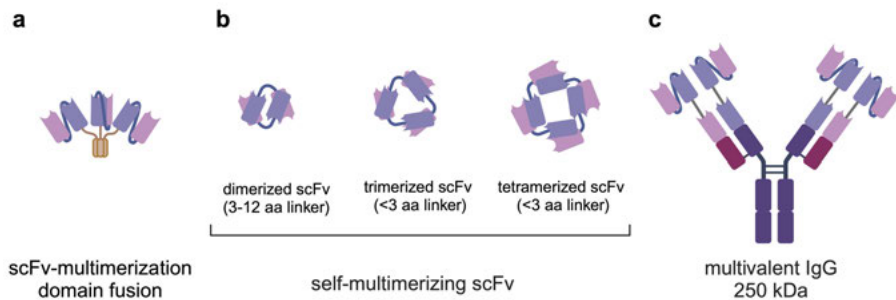

Figure 2. Schematic illustration of multivalent antibodies and antibody fragments. (a) scFv fused C-terminally to a multimerization domain. (b) scFv with internal linkers shorter than 12 amino acids dimerize, trimerize or tetramerize. (c) scFv fused to the N-terminal end of the variable domains of an IgG antibody.

Blood-brain barrier (BBB)

The blood-brain barrier (BBB) is built up of endothelial cells, pericytes, and astrocyte endfeet (Fig. 3) and tightly regulates the entrance of molecules, ions and cells into the brain, in order to protect it from potentially harmful compounds. The BBB endothelium is particularly tight compared to other endothelia as it is sealed by tight junction proteins such as Occludin, Claudins, and junction adhesion molecules, which prevent the passive passage of polar molecules through the paracellular space [26–28]. Despite lipophilic molecules with a molecular weight below 400 kDa, which can diffuse passively through the

BBB [29], all other molecules can only move across the BBB by transporter-mediated or vesicular transport. Specialized solute carrier transporters, on the one hand, facilitate the transport of molecules including amino acids, carbohydrates, hormones, fatty acids, vitamins and nucleotides as well as the efflux of several metabolites across the cell membranes [27]. Vesicular transport, or transcytosis, on the other hand, is characterized by the uptake of macromolecules into vesicles on the endothelial surface and the release of the cargo on the basolateral side of the endothelium by fusion of the vesical membrane with the cell membrane. The two transcytosis mechanisms that are present on the BBB are 1) adsorptive-mediated transcytosis with cationic peptides or proteins attaching unspecifically by charge to the endothelial surface [30], and 2) receptor-mediated transcytosis with proteins such as insulin and transferrin binding specifically to a receptor present on the endothelial cell surface and subsequently being transcytosed across the BBB.

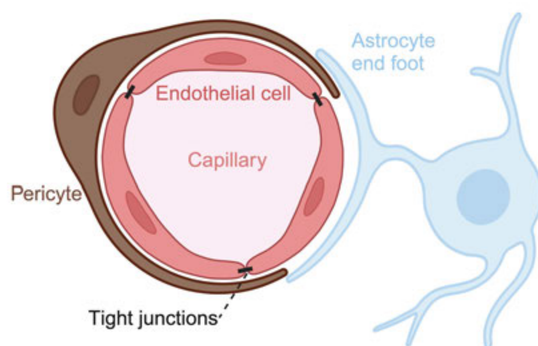

Figure 3. Schematic representation of the BBB. The BBB is built up of endothelial cells enclosing the capillary lumen, sealed with tight junctions, and surrounded by pericytes and astrocyte endfeet.

Protein-drug delivery across the BBB

Despite the strong limitations of substance transport into the brain, the BBB endothelium also represents a great opportunity for brain-target drug delivery due to its large blood-brain interface covering a total area of approximately 12-20 m^2 in an average adult [26,29], which offers a large surface on which drug transport can take place and the possibility to deliver a drug to almost any part of the brain.

The size and chemical properties of any therapeutic protein largely prevent its passive uptake into the brain. The proportion of an intravenously injected full-sized antibody reaching the brain interstitium has been estimated to less than ~0,1% [31–33]. Various strategies have been explored to enable protein drug delivery to the brain, most commonly by hitchhiking on existing transport mechanisms. Brain uptake mediated by cell penetrating peptides (CPP) has

been described with cationic peptides that are presumably taken up by adsorptive-mediated transcytosis. However, due to the unspecific nature of CPP-mediated uptake mechanisms, they are not specifically targeting the brain and may lead to undesired side effects due to a distribution in peripheral tissues [34,35]. More brain-specific protein drug uptake is by receptor-mediated transcytosis , targeting receptors that are highly expressed on the BBB endothelium, such as the insulin receptor [36], CD98hc [37], and the transferrin receptor (TfR).

Transferrin receptor (TfR)-mediated transcytosis

The transferrin receptor is a dimeric transmembrane protein comprising a large extracellular domain, which is connected through a stalk to its transmembrane domain, followed by a small intracellular domain. Two different versions of TfR, TfR1 and TfR2, are known. TfR1 is expressed ubiquitously, while TfR2 is expressed predominantly on liver cells and erythroid cells in the bone marrow. Due to its expression on BBB endothelial cells, TfR1 has gained much interest as potential protein drug transporter into the brain [38]. Nevertheless, since TfR1 is also highly expressed in tissues other than the BBB endothelium, targeting it can potentially give rise to undesired drug accumulation and side effects in those tissues as well as a reduced drug efficacy due to binding of the drug to TfR in blood. In this work the focus is only on TfR1.

The physiological function of TfR is the delivery of iron to cells and organs, which is mediated through the iron-binding protein transferrin (Tf). In blood at pH 7,4, iron binds with high affinity to Tf, forming holoTf. Binding of two holoTf molecules near the stalk region on a TfR dimer induces clathrin-dependent endocytosis of the complex [39,40]. The mechanism of how iron is transported across the BBB endothelium and released to the brain interstitium is not fully understood and controversially discussed. For TfR-trafficking in erythrocytes, the so called canonical Tf-TfR cycling pathway has been described [41], which has also been proposed as a suitable model for the iron uptake across the BBB. The model claims that iron detaches from Tf in the late endosome due to the decreased pH and that iron is subsequently transported through the divalent metal transporter 1 (DMT1) into the cytosol. Alternative models suggest either the basolateral exocytosis of only iron that has detached from Tf in the low endosomal pH, or the exocytosis of the whole TfR-holoTf complex where iron or alternatively holoTf, is released by factors other than low pH, while TfR-apoTf complexes are thought to be retro-endocytosed to be recycled to the apical endothelial surface [42–44]. As an additional hypothesis, exosomal release of holoTf from the BBB endothelial cell into the brain has been suggested [45].

Consequently, the details of how antibodies are transported across the BBB remain elusive too. Similarly to the release of iron from TfR-holoTf in the low pH of late endosomes, a reduced affinity to TfR at low pH has also been

suggested to be advantageous for BBB-shuttles, for which the rat antibody 8D3 is an example [46] (Fig. 4). Other factors that are considered important for an efficient TfR-mediated transcytosis are a relatively low affinity [47,48] and a monovalent mode of binding to TfR [49]. Both, high affinity and bivalent binding to TfR instead can cause retention or lysosomal degradation of endocytosed constructs in the BBB epithelium or induce clustering of TfRs on the epithelial cell surface which reduces the transcytosis efficacy. The effect of TfR clustering through bivalent TfR-binders has been observed to be particularly pronounced at high antibody treatment doses [18,49].

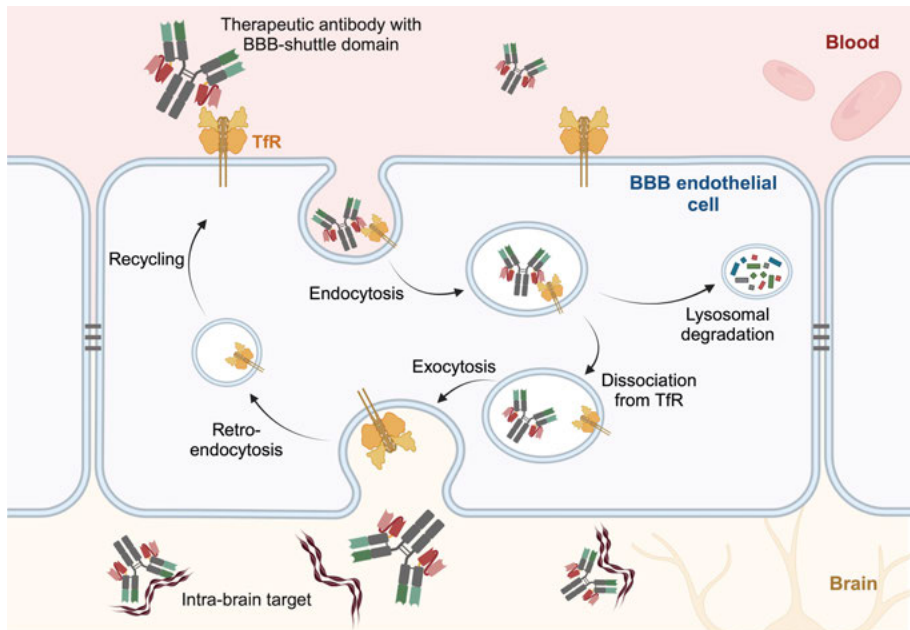

Figure 4. Schematic illustration of a hypothetical mechanism for the transcytosis of the bispecific antibody RmAb158-scFv8D3 through the BBB endothelium. The antibody binds with its BBB-shuttle domain scFv8D3 to the TfR on the apical side of BBB endothelial cells. After endocytosis of the complex, the antibody dissociates from TfR and is released by exocytosis to the brain interstitium, while the TfR is recycled to the apical endothelium surface.

Various designs for TfR-targeting protein-drugs exist, where a TfR-binding moiety, such as antibody fragments, other proteins or peptides are fused to a therapeutic protein to induce TfR-mediated transcytosis into the brain [47,49–54]. A common strategy to produce bispecific antibodies, which bind monovalently to each target is the knobs-into-holes technology. Knobs-into-holes antibodies are based on modifications in the Fc chain, which inhibit the pairing of two identical heavy chains leading to hetero-dimer formation between two different heavy and light chain pairs. Downsides with the knobs-into-holes

design are the reduced production yield compared to homodimeric antibodies, due to mispairing antibodies. Furthermore, knobs-into-holes antibodies usually also bind their therapeutic target monovalently which often results in a lower efficacy than bivalent binding to the therapeutic target. One bispecific antibody binding the TfR monovalently but its therapeutic target bivalently has been described [53]. Here the TfR-binding moiety is included in one of the two different Fc domains, where the hetero-dimeric structure is again achieved by a knobs-into-holes design [53]. Alternatively, the bispecific antibody RmAb158-scFv8D3, which does not depend on knobs-into-holes pairing, also shows strongly enhanced brain uptake while still offering bivalent binding to its therapeutic target. For Rmab158-scFv8D3 it was hypothesized, that the placement of the scFv8D3 in the RmAb158 light chain C-termini limits bivalent TfR-binding (Fig. 4).

Parkinson's disease

Parkinson's disease (PD) is the second-most prevalent neurodegenerative disease with globally over 8.5 million cases estimated by the WHO in 2019 [55]. It is characterized by the progressive degeneration of dopaminergic neurons of the substantia nigra in the midbrain causing motor impairments such as resting tremor, muscle rigidity, postural instability and bradykinesia as well as non-motor symptoms such as speech difficulties, sleep disturbances, loss of olfaction, impaired cognition and dementia in later disease stages [56].

Microscopically visible protein inclusions, in PD patients' brains were first described by Friedrich Lewy in 1912. These so called Lewy bodies (LB) are mainly composed of aggregated alpha-synuclein (αSyn) [57] and are located predominantly in the substantia nigra, but can be found during later disease stages also in other brain areas such as the olfactory bulb, limbic system, and the neocortex [58]. However, the pathological significance of LB has been questioned and LB are nowadays considered inert deposits for aggregated αSyn protecting neurons from its toxic effects [59]. In support of this hypothesis is the poor correlation of the severity of the patient's PD symptoms and the amount of LB in the brain, as well as the observation of LB-containing neurons appearing more healthy than adjacent LB-free neurons [60,61]. Besides PD, there are other diseases summarized as α-synucleinopathies, characterized by the accumulation of aggregated αSyn in the brain, such as Dementia with Lewy bodies and Multiple system atrophy, which mainly differ in the affected cell types and brain region [62]. Above that, protein aggregation is a common feature to other neurodegenerative diseases where typically the aggregation of one specific protein is associated with the disease's progression, including amyloid-beta (Aβ) and Tau in Alzheimer's disease, and huntingtin in Huntington's disease [63]. Further, co-pathologies with the aggregation of different of these proteins is not rarely observed [64].

The causes of PD are mostly unknown but age is considered the main "risk factor". Environmental factors such as exposure to pesticides, heavy metals and traumatic brain injuries have been discussed to also play a role in the propensity of developing the disease [65,66]. About 10% of all PD cases can be ascribed to inherited mutations. The most prominent of those mutations are located in the genes coding for αSyn (SNCA), Parkin (PRKN), PTEN-induced kinase 1 (PINK1) and Leucine-rich repeat kinase 2 (LRRK2) [65,67], which usually cause a high risk to develop PD and are often associated with an early disease onset.

A symptomatic treatment of PD is available since 1967, where the precursor of dopamine, L-3,4-dihydrophenylalanine (L-DOPA) is used to compensate for the dopamine deficiency but it cannot halt the progression of neuronal loss and long-term treatment comes with side effects such as dyskinesia and motor fluctuations [68].

α-Synuclein (αSyn)

αSyn is a protein built up of 140 amino acids, which is highly abundant within presynapses of the central nervous system [69]. The physiological role of αSyn is not entirely understood, but it is believed to be involved in neurotransmitter release, synaptic vesical release and recycling, and neuronal plasticity [70–73]. Furthermore, αSyn has been suggested to be involved in the transport of fatty acids between the cytosol and the lipid membrane [74]. Knock-out of αSyn in mice has not been observed to cause severe impairments, probably due to functional redundancy between members of the synuclein protein family, where β- and γ-synuclein are likely able to replace most αSyn functions [75].

Under physiological conditions, αSyn is found in an equilibrium between a cytosolic, unfolded state and a membrane-bound, helical state [76–78]. The primary structure of αSyn can be divided in three regions with distinct characteristics: The positively-charged N-terminal region (residues 1-60), the highly hydrophobic central region (residues 61-95), known as the non-amyloid-β component (NAC) region, and the negatively-charged C-terminal region (residues 96-140) (Fig. 5). The interaction of αSyn with phospholipid membranes is mediated through seven imperfect repeats, located in both N-terminal and NAC region, that form a continuous or broken amphipathic helix spanning residues 1-90 [79,80]. Its ability to interact with membranes is considered critical for both the physiological functions of αSyn monomers as well as for the membrane-associated harmful effects of toxic oligomers [81]. The first 25 residues of αSyn appear to be responsible for the initiation of membrane binding through helix formation, before the helix conformation extents to its full length [82]. Disturbance of the membrane-binding through e.g. a high αSyn concentration relative to the membrane surface or an altered lipid composition, so that only the first part of the helix will form and bind to the membrane, leaves the NAC region exposed which can initiate αSyn aggregation [83,84].

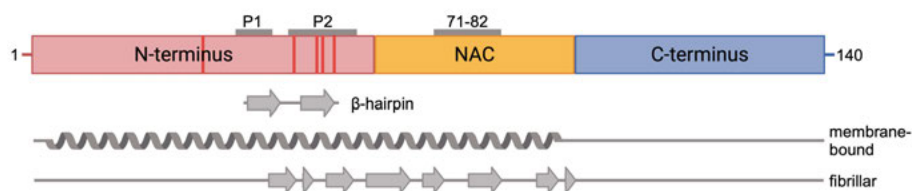

Figure 5. Primary and secondary structures of αSyn. Indicated are N-terminal (red), NAC (yellow) and C-terminal (blue) regions, positions of single point mutations in familial PD cases (red lines), P1 and P2 'master regulator' segments [85], 71-82 residue segment crucial for fibrillation [86], β-hairpin suggested to initiate oligomerization [87,88], α-helical region in membrane-bound state [80], and β-strands in fibrillar state [89].

The NAC region is considered the aggregation-prone region as it forms the β-sheet-rich core of αSyn fibrils and a peptide consisting of the NAC region alone is able to fibrillate [90]. Meanwhile, the deletion of a 11-residue segment (residues 71-82) from the central NAC has been reported to impair αSyn fibrillation [86]. In the full-length protein, the C-terminal region exhibits a protective role against spontaneous aggregation through long-range interactions with the N-terminus and the NAC [91]. Truncations or shielding of the negative charges in the C-terminus by e.g. calcium [92], metal ions [93] or low pH [94] interfere with these long-range interactions and accelerate fibrillation [95]. Additionally, two segments in the N-terminal region sometimes referred to as master regulator P1 (residues 36-42) and P2 (residues 45-57) have been identified to play a regulatory role in αSyn fibrillation in that their blockage or deletion abolished fibrillation [85,96].

All single point mutations in αSyn that are associated with familial cases of PD are located in the N-terminal region and include A30P [60], E46K [97], H50Q [98], G51D [99], A53T [100], A53V [101] and A53E [102]. These mutations have shown to disturb the protective long-range interactions between N- and C-terminus, to interfere with fibril-stabilizing contacts, or to create new, possibly oligomer-stabilizing contacts [102,103]. Hence, only the mutations E46K, H50Q, A53T and A53V cause faster fibrillation than wild-type (wt) αSyn. In contrast, A30P, G51D and A53T show slower fibrillation kinetics but are thought to remain longer in an oligomeric phase, where the longer exposure of cells to toxic oligomers may explain the pathological mechanism of these mutants [102].

αSyn aggregation

The aggregation of αSyn starts with the misfolding of αSyn monomers into a self-assembly-prone conformation. A β-hairpin conformation (residues 38-53) involving the above-mentioned regulator sequences P1 and P2 in the αSyn N-terminus has been suggested to be the initial misfolded conformation [87,88]. The assembly of several misfolded αSyn monomers leads to the formation of small αSyn oligomers that serve as nuclei for further aggregation [104] (Fig. 6).

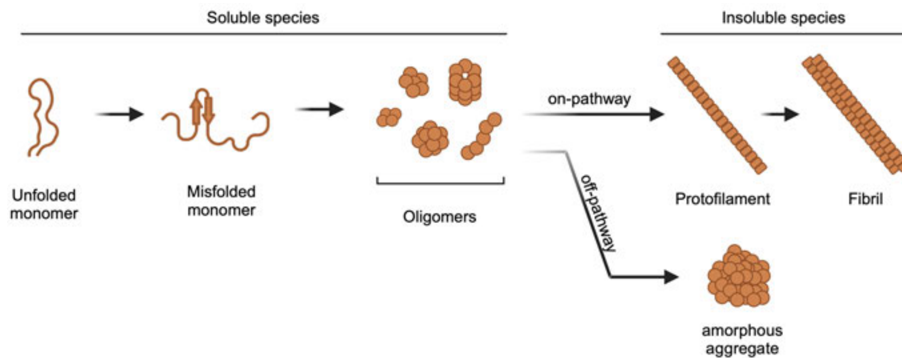

Figure 6. αSyn aggregation cascade. αSyn monomers are unstructured and adopt some degree of secondary structure when they misfold. Misfolded αSyn assemble into oligomers which can adopt a higher degree of β-sheet structure and convert into fibril seeds ('on-pathway') or grow into amorphous aggregates ('off-pathway').

αSyn oligomers

The term αSyn oligomers describes a rather poorly defined group of soluble aggregates for which various sizes, shapes, and degrees of secondary structure content have been reported [105]. Spherical, annular and elongated αSyn oligomers have been described [81,106,107]. αSyn oligomers are considered to be predominantly disordered but have some degree of secondary structure. Also oligomers with a high α-helix content have been reported [108], but most commonly they are described with a β-sheet structure content which increases with increasing oligomer size but is at any time lower than in fibrils [109]. In contrast to the parallel cross-β sheet structure of fibrils, αSyn oligomers are characterized by an anti-parallel β-sheet structure [110]. This anti-parallel β-sheet structure of oligomers might be represented by the β-hairpin conformation (residues 38-53) [88]. To initiate the formation of fibrils, oligomers must undergo a conversion from their anti-parallel [110] into a parallel β-sheet arrangement [104,111]. However, not all oligomers convert into fibrils but may remain as off-pathway oligomers or form amorphous aggregates [112,113]. The transient nature of αSyn oligomers makes the characterization of the full spectrum of oligomer species and their individual roles in neurotoxicity and seeding difficult. Chemically stabilized [114–116] or metastable oligomers [109,117,118] are widely used for studying the structure and role of αSyn oligomers but those methods likely miss out on small oligomers that occur early in the aggregation pathway.

αSyn fibrils

αSyn fibrils typically consist of two protofilaments in which the individual αSyn subunits are stacked along the fibril axis. Most of the NAC region and part of the N-terminus contain multiple β-strands which form the protofilament core, while the C-terminus remains unstructured [89]. The specific folding

pattern of a β-strands within a fibril depends on the fibrillation conditions [119–123], but during fibril elongation, the folding pattern of the elongating fibril is preserved by it acting as a template on the newly incorporated monomers [124]. Fibril elongation occurs by monomer addition, where the residues 74-82 have been suggested to form the initial contact between the fibril end and the monomer [89], upon which the monomer undergoes structural conversion to adopt the same fold as the templating fibril [119,125,126].

Secondary processes that accelerate aggregation are fibril fragmentation and secondary nucleation. Fibril fragmentation simply increases the number of fibril ends at which elongation can simultaneously take place. In contrast, secondary nucleation describes the surface-catalyzed formation of aggregates from monomers that transiently adhere to the lateral fibril surface. While fibril elongation has been observed to be the dominant process of fibril growth at neutral pH, secondary nucleation has been shown to be the dominant process at slightly acidic pH [127].

αSyn cell-to-cell transmission

Various mechanisms have been proposed for how αSyn oligomers and fibrils are transmitted between cells and thereby spread the pathology. Those mechanism include exocytosis and receptor-mediated endocytosis, exosomal release, tunneling nanotubes, heparan sulfate proteoglycan (HSPG)-mediated uptake or membrane penetration [128]. However, the type and efficiency of transmission between cells varies strongly between different αSyn aggregate species and different cell types [129]. In the context of the endocytotic uptake of αSyn aggregates, αSyn has shown the ability to inhibit its lysosomal degradation [130] and disrupt the endosomal or lysosomal vesicle to escape into the cytosol where it can seed further aggregation [131,132]. The exposure of αSyn to the acidic pH in the endolysosomal compartment may also play a role in accelerating aggregation [127].

αSyn toxicity

The most neurotoxic effects of αSyn aggregates are considered to stem from αSyn oligomers [133,134], while toxic effects seen with fibrils likely originate from their secondary nucleation activity [104,135]. Among the αSyn aggregate-associated effects that have been observed on cells are membrane permeabilization [136], calcium influx [106], dysfunction of organelles such as of mitochondria and the endoplasmic reticulum [137,138], dysfunction of lysosomal and proteasomal degradation systems [130,139], neuroinflammation [140,141] and apoptosis [135]. Those effects have been observed with either intracellular or extracellular αSyn aggregates and have been attributed to e.g. direct insertion of the αSyn aggregates into membranes but also to αSyn-mediated activation of

receptors such as NMDA receptors on neurons [142] and toll-like receptors 2 and 4 on astrocytes and microglia cells [140,141].

A variety of different types of αSyn oligomers, prepared by a variety of different methods have been described but it is difficult to identify a clear link between their structures, toxicities and seeding-competencies [105]. Together with studies on other amyloid proteins it has been suggested that an intermediate content of β-sheet structure, a high degree of solvent-exposed hydrophobicity and the ability to interact with membranes with the N-terminus may be responsible for toxic effects through direct membrane contact [143]. However, it is likely that there are different toxic αSyn oligomeric species which may exert their toxic effects through different mechanisms and structural features.

Therapeutically targeting αSyn

Up to now, there is no disease-modifying treatment for PD or other α-synucleinopathies available. With its central role in the disease progression, αSyn is an attractive therapeutic target and approaches that have been explored to halt or reverse the disease progression are described for reducing αSyn synthesis, increasing its degradation, inhibiting its aggregation or blocking its inter-neuronal transmission [144]. To remove extracellular toxic αSyn aggregates from the brain, immunotherapeutic approaches with the antibodies' high target specificity, high affinity and long half-lives are here of particular interest [145]. Additionally, the recent successes in the field of Alzheimer's disease with the approval of the therapeutic antibody Lecanemab targeting Aβ protofibrils are further encouraging for the immunotherapy development in the PD field. However, all five αSyn aggregate-targeting antibodies that have been in clinical trials have failed to show therapeutic effects [146]. The major challenges that have likely limited the therapeutic efficacy of anti-αSyn antibodies are 1) the small proportion αSyn aggregates that is accessible extracellularly to antibodies, 2) the lack of selectivity for the actually toxic aggregates, 3) the need of a BBB-transporter to enable antibodies to bind intra-brain targets [146].

Methodology

Protein design and production

All proteins produced in this work were engineered using recombinant DNA technology, which allows to create protein constructs with new functionalities by fusing different proteins, protein domains or peptides of known sequences [147]. The nucleotide sequence of a construct was cloned into the pcDNA3.4 vector and transfected into Expi293 cells as a transient expression system. Each construct was designed with a N-terminal signal peptide for secretion into the cell medium from where the protein could be easily purified by affinity purification [148].

Different IgG antibody formats were recombinantly produced in this work including simple full-length antibodies, multivalent antibodies with scFv fused to the antibody's N-terminals [149] (**Paper I**), bispecific antibodies with scFv fused to the antibody's light chain C-terminal (**Paper II**), or scFv fused to a single-chain Fc (scFc) [18] (**Paper III**) (Fig. 7). In contrast, Fab fragments that were used in **Paper I** were generated by papain digestion of a full-length IgG, which cleaves in the hinge region resulting in the separation of the two Fab fragments and the Fc.

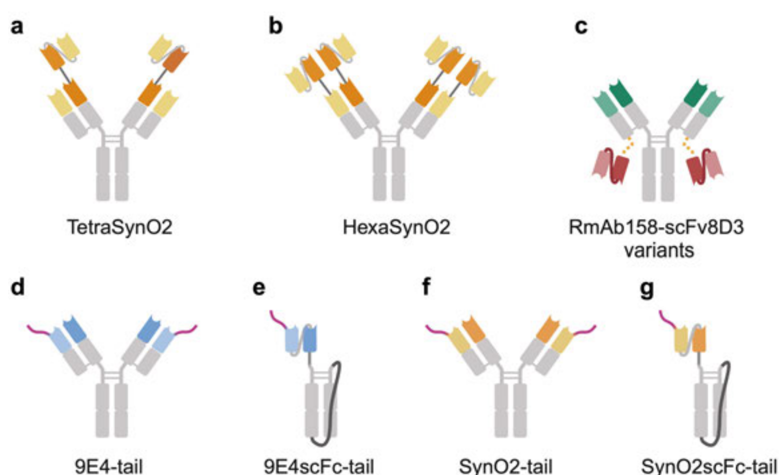

Figure 7. Schematic illustration of antibody construct designs produced in this thesis. (a) TetraSynO2 (b) HexaSynO2 (c) RmAb158-scFv8D3 with varying linker lengths (d) 9E4-tail (e) 9E4scFc-tail (f) SynO2-tail (g) SynO2scFc-tail.

Protein structure prediction

Protein structure predictions using AlphaFold2 (AF2) were used in **Paper III** and **Paper IV** to aid the protein design and to draw structure-based conclusions from the obtained experimental results. AlphaFold has revolutionized the field of amino acid sequence-based protein structure prediction with its high prediction accuracy based on a deep-learning algorithm that involves both biological and physical knowledge [150]. However, AF2 has been shown to have limited accuracy in predicting intrinsically disordered proteins or disordered regions [151].

Preparation of αSyn aggregates

Different protocols were used to prepare αSyn aggregates in this work. Aiming to produce αSyn oligomers in **Paper I**, αSyn was incubated in the presence of 4-hydroxy-2-nonenal (HNE) which has been reported to produce stable oligomers [152]. In our hands, however, the HNE-mediated aggregation produced soluble, but large and filament-like structures. αSyn fibrils were prepared in both **Paper I** and **Paper IV** by incubation in PBS at 37 °C shaking for 7 days. The same conditions applied to αSyn mutants designed in Paper IV lead to the formation of small αSyn oligomers.

Affinity determination

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a widely used assay to characterize protein-protein interactions and to quantify specific proteins in a sample. In this work, three different types of ELISAs were used to determine the binding strength of the engineered antibodies to their targets.

Indirect ELISA

The indirect ELISA is the simplest ELISA set-up where the target protein is immobilized directly as a coating to the assay plate and a serial dilution of the antibody whose binding strength is to be determined is incubated on the coating (Fig. 8 a). In a next step, a secondary, horseradish peroxidase (HRP)-conjugated antibody that binds the constant region of the primary antibody is added. Alternatively, if the primary antibody was biotinylated, HRP-conjugated streptavidin that binds to biotin can be added. Finally, the addition of the HRP substrate tetramethylbenzidine (TMB) results in a colorimetric signal, where the signal intensity in relation to the concentration of the primary antibody give conclusion about the antibody's binding strength. The indirect ELISA was used in **Paper I**, **Paper II** and **Paper III** to determine the antibodies' binding strengths to their targets, i.e. αSyn aggregates or murine TfR

(mTfR). In **Paper I**, we aimed to compare the binding strength of a full antibody and a Fab where the lack of the Fc would cause a much weaker binding of the secondary detection antibody to the Fab compared to the full antibody. Therefore, both the full antibody and the Fab were biotinylated for detection with HRP-conjugated streptavidin. To ensure that both full antibody and Fab had a similar degree of biotinylation, the number of biotins per protein was further validated by mass spectrometry. In **Paper II**, we were particularly interested in detecting small differences in the degree of monovalent and bivalent binding of the antibodies to mTfR, where the coating density of mTfR played a major role. Since the antibodies' ability to bind mTfR monovalently or bivalently was primarily dependent on the distance of available mTfR subunits, we chose a relatively high TfR concentration for the coating in order to allow bivalent binding as much as possible.

Sandwich ELISA

The sandwich ELISA differs from the indirect ELISA in that the target protein is not applied as coating but is captured by an antibody which is immobilized to the assay plate in a first step (Fig. 8 b). This set-up can be of advantage e.g. if a certain orientation of the target is of importance, to block a specific epitope, or to stop the target from self-associating. In **Paper I**, a sandwich ELISA was carried out to test the cross-reactivity of anti-αSyn antibodies with Aβ42 protofibrils, where this set-up reduces the risk of Aβ42 protofibrils to assemble into larger aggregates. In **Paper II**, a sandwich ELISA was used to quantify the amount of antibodies that transcytosed across an *in vitro* BBB model.

Inhibition ELISA

An inhibition ELISA set-up was chosen in **Paper I** to detect differences in the binding strength between very high-affinity bi- and multivalent anti-αSyn antibodies, where an indirect ELISA was not sensitive enough to detect differences. The inhibition ELISA further allows to identify the binding preference of an antibody to one over another target. Here, antibodies are pre-incubated with a serial dilution of different targets or target species in solution, before these pre-incubated solutions are transferred to a plate pre-coated with one type of target (Fig. 8 c). Stronger binding to the target in solution thus is expected to result in a lower binding signal on the plate and vice versa.

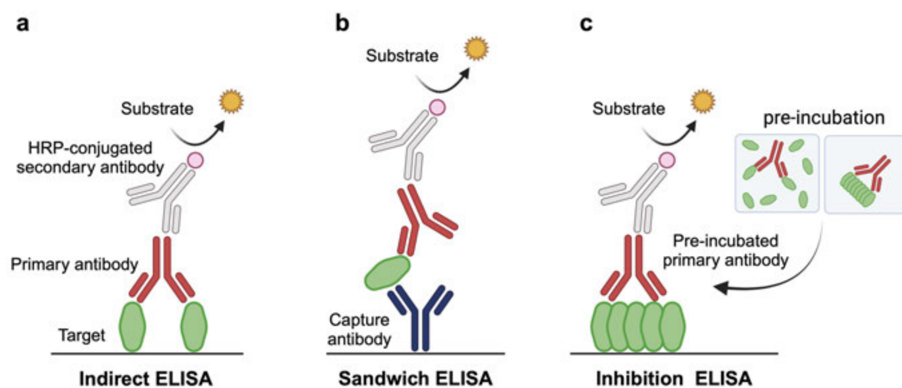

Figure 8. Schematic illustration of ELISA set-ups to determine antibody binding strength. (a) Indirect ELISA. (b) Sandwich ELISA. (c) Inhibition ELISA.

LigandTracer

A more detailed kinetic characterization of protein-protein interactions than by ELISA can be accomplished by real-time interaction analysis using e.g. a LigandTracer instrument. In this method, the target protein or cells are immobilized in a target area on a Petri dish which is placed on a tilted, rotating platform in the instrument (Fig. 9). During at least two association phases, iodine-125 (^{125}I)-labelled antibodies are incubated at increasing concentrations. Subsequently, unbound antibodies are removed and the dissociation of antibodies is measured. The interaction of the antibodies with the target is recorded by a low-energy gamma detector. Kinetic parameters such as the association rate (k_a), dissociation rate (k_d) and the affinity (K_D), are calculated by fitting the interaction curve with a suitable kinetic model. In contrast to Surface plasmon resonance, which is a more common method to determine the kinetics of protein-protein interactions, LigandTracer allows to measure the dissociation over many hours which is advantageous for the kinetic evaluation of very strong binders with very slow dissociation rates [153]. LigandTracer was used in **Paper I** and **Paper II** for the kinetic evaluation of the interactions between antibodies and αSyn aggregates or mTfR, respectively.

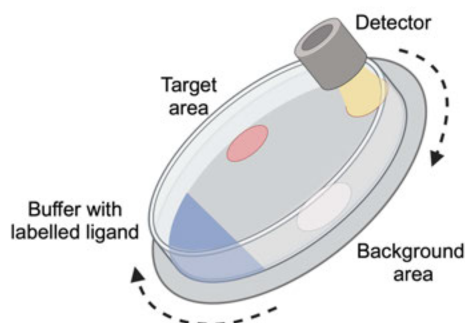

Figure 9. Schematic illustration of LigandTracer set-up. A petri dish is coated with a target protein in the target area and placed on a tilted platform in the LigandTracer instrument. A buffer solution containing ^{125}I-labelled ligand is added. Through rotation of the platform, the target area and the uncoated background are alternately incubated with the ligand solution. A low-energy gamma detector above the upper side of the platform measures the radioactivity bound to the target area and background area respectively.

Characterization of protein stability and structure

Different biophysical methods were employed in this work to study the proteins' stability and structural features.

In **Paper I**, **Paper II** and **Paper III**, the antibodies' thermal stability was determined by **differential scanning fluorimetry (DSF)**. DSF makes use of the intrinsic fluorescence of tryptophan and tyrosine residues which is dependent on the residues' local environment and thus on the protein's folding. In a thermal shift assay, a protein's unfolding can thus be monitored by DSF and inflection temperatures, which represent major unfolding events, can be determined [154]. A lower inflection temperature indicates a lower thermal stability of a protein. In **Paper I** and **Paper II**, the antibodies were additionally characterized by **Size exclusion chromatography (SEC)**, which is a common method to detect protein aggregates in a purified protein sample. SEC is based on the different retention times of differently sized particles while flowing through the porous medium of a SEC column. **Mass photometry**, a method that can relatively accurately determine a molecule's mass was used in **Paper II**, **Paper III** and **Paper IV** to determine the mass distribution of mTfR, antibodies and αSyn aggregates, respectively. Mass photometry is based on the light scattering intensity that a particle generates when it touches the glass surface of the instrument. The particle's mass can be derived as it is directly proportional to its light scattering intensity [155]. However, mass photometry is limited to particles above ~20 kDa due to too weak scattering signals from particles below that size. In **Paper IV**, we therefore also used **dynamic light scattering (DLS)** to characterize the monomeric αSyn mutants by their hydrodynamic radii. DLS measures the diffusion coefficient of particles by the

particles' light scattering fluctuation rate. DLS has, however, a low size resolution and its very high sensitivity for large particles makes it easily affected by particulate contaminants such as dust [156].

The overall morphology of αSyn HNE aggregates in **Paper I** and of αSyn fibrils in **Paper IV** was investigated by **transmission electron microscopy (TEM)**, which offers a better resolution than most other microscopy techniques but may affect the aggregate structure due to dehydration, the immobilization on the charged grid surface and the requirement of exposing the protein to metal salts such as uranyl acetate for negative staining [157]. Additionally, **circular dichroism (CD)** was used in **Paper IV** to determine the overall secondary structure content of both soluble and insoluble αSyn aggregates. CD is based on the principle that the peptide bonds of the protein backbone absorb circularly polarized light (typically 190-260 nm) differently depending on their secondary structure involvement, but it only resolves the average secondary structure content of a sample [158].

αSyn aggregation assays

The fibrillation propensity of αSyn mutants was assessed in **Paper IV** through aggregation assays monitoring the fluorescence intensity increase of the dye Thioflavin T (ThT). ThT binds specifically to the cross-β structure of amyloid fibrils, a state in which it emits a strong fluorescence at ~480 nm upon excitation at 450 nm, whereas free ThT in solution exhibits very low fluorescence [159]. The binding of ThT to amyloid fibrils has been suggested to be driven by hydrophobic and aromatic interactions in a groove along the fibril's axis [160].

Variations in the aggregation assay conditions such as different monomer concentrations, shaking vs. quiescent incubation, the addition of aggregation seeds, a slightly acidic pH or the addition of a low concentration of sodium dodecyl sulfate (SDS) can be used to assess specific characteristics of the fibrillation such as the contribution of primary nucleation, secondary nucleation or fibril elongation rate to the overall fibril growth [161]. In **Paper IV**, the basic aggregation assay set-up was performed with 40 µM αSyn monomers with 40 nM sonicated preformed fibrils (PFF) as aggregation seeds. For co-aggregation studies, additional 4 µM αSyn mutant monomers were added to 40 µM αSyn wild-type monomers and 40 nM PFF. The major advantage of including PFF seeds in the set-up is to speed up the assay by reducing the lag phase. A third assay set-up involved SDS, which can mimic the impact that negatively charged lipid membranes have on αSyn aggregation. At the right concentration, SDS accelerates αSyn aggregation without the need for PFF seeds [162,163].

Cell-based assays

In-Cell BBB-Trans assay

In **Paper II**, an *in vitro* BBB model was used to evaluate the transcytosis efficiency of different antibodies across a brain endothelial cell layer. The In-Cell BBB-Trans assay was developed in our group and is based on a "pulse-chase" experimental set-up using mouse brain endothelial cells grown on a porous cell culture insert [164] (Fig. 10). In contrast to more elaborate BBB models involving e.g. multiple cell types or flow in a microfluidic set-up, our model may not exhibit an impermeable cell layer with high transendothelial electrical resistance [165], but it has proven to reliably predict the degree of TfR-mediated uptake of therapeutic antibodies into the mouse brain [164].

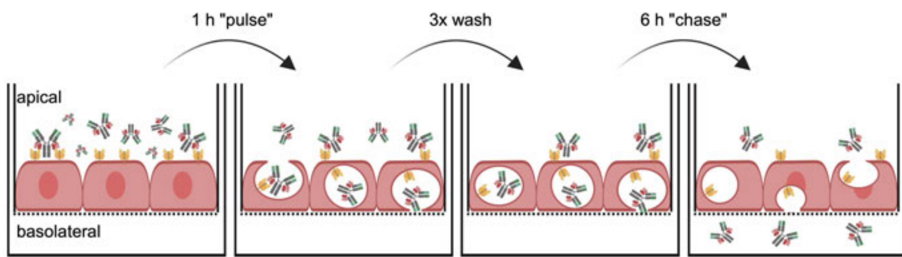

Figure 10. Schematic illustration of the In-Cell BBB-Trans assay [166]. Differentiated cEND cells grown on porous cell culture inserts are treated with antibodies for 1 h ("pulse") during which the antibodies bind to TfR on the apical epithelial surface and are endocytosed. Three washes remove unbound antibodies. Antibodies present in the apical and basolateral chamber after 6 h incubation ("chase") are quantified by a sandwich ELISA.

Toxicity assays

In **Paper IV**, the effects of αSyn aggregates on neuronal cells was investigated using the neuroblastoma cell line SH-SY5Y which is a widely used cell model for studying neurodegeneration *in vitro* [167]. Undifferentiated SH-SY5Y cells were treated with aggregated αSyn for 24 h and toxic effects were assessed based on the cells' metabolic activity and the proportion of early and late apoptotic cells. The metabolic activity was measured using an MTT assay where the readout depends on the enzymatic reduction of a tetrazolium salt into the colored formazan [168]. The proportion of apoptotic cells was assessed by flow cytometric analysis of Annexin V and 7-AAD-stained cells. Annexin V binds to phosphatidyl serin which only gets exposed on the cell surface upon entering an early apoptotic state. 7-AAD intercalates into DNA strands but is membrane-impermeable dye, hence only stains cells with membrane defects indicating a late apoptotic state [169].

Aims

The overall aim of this thesis was to engineer improved antibody-based drugs for the treatment of αSyn pathology by increasing the antibodies' binding strength to pathologically relevant αSyn aggregate species and by enhancing the brain delivery of therapeutic antibodies.

In **Paper I**, we engineered two multivalent αSyn-specific antibodies where the higher number and the shorter distance of binding sites was aimed to enhance the antibodies' binding strength to a wide range of soluble αSyn aggregates.

In **Paper II**, we investigated whether the TfR-mediated transport of the symmetric, bispecific antibody RmAb158-scFv8D3 across the BBB can be enhanced by optimizing the linker length between the therapeutic antibody and its TfR-binding scFv.

In **Paper III**, we aimed to enhance the avidity of antibodies to αSyn aggregates by engineering antibodies with a negatively charged peptide tail.

In **Paper IV**, we designed αSyn mutants with the aim to produce stable, small oligomers and characterized their aggregation propensity and cytotoxicity.

Summary of investigations

Paper I

We engineered the tetravalent TetraSynO2 and the hexavalent HexaSynO2 antibody based on the parental antibody SynO2. The higher valency of these antibodies was achieved through the fusion of SynO2scFv to the antibody's N-termini. The binding strength of these new antibodies to αSyn aggregates was evaluated by ELISA and LigandTracer and showed a greatly enhanced binding strength of these multivalent antibodies compared to the parental bivalent antibody. In particular, we showed that the dissociation of the multivalent antibodies was slower due to an increased proportion of antibodies binding bi- or multivalently to the target. These findings show that increasing an anti-αSyn antibody's valency can indeed reduce its dissociation from αSyn aggregates, bearing the potential of more effectively capturing and clearing αSyn aggregates in a therapeutic context.

However, the multivalent SynO2 antibodies were characterized by low expression yields compared to a previously reported multivalent antibody [149], suggesting a low potential for developability of the multivalent SynO2 antibodies, and further indicates that this antibody design may not be directly translatable to any antibody sequences. Further, we were not able to test the hypothesis that the multivalent antibodies can bind smaller αSyn aggregates than the parental antibody due to the lack of small stable αSyn oligomers.

Paper II

Previous studies had shown that despite RmAb158-scFv8D3 exhibiting a much better brain uptake than other antibody formats of bivalent TfR-binders, this antibody was still able to bind TfR bivalently. As bivalent binding to TfR is considered to result in lower brain uptake than monovalent binding, we attempted here to increase the steric hindrance within the antibody to reduce its ability of bivalent TfR-binding to a minimum. By drastically shortening the linker that connects the scFv8D3 with the RmAb158 light chain, we achieved an almost two-fold better transcytosis in the *in vitro* BBB model compared with the original bispecific antibody design. Together with the kinetics of the RmAb158-scFv8D3 variants binding to TfR, these results suggest that none of the new RmAb158-scFv8D3 variants had a decreased ability of binding bivalently to TfR. Instead, we ascribe the improved *in vitro* BBB transcytosis

of the shorter linker variant to a reduced ability of cross-linking TfR on the cell surface, which otherwise limits the transcytosis efficiency.

Paper III

One mechanism of cell-to-cell spreading of αSyn aggregates has been suggested to be dependent on the interaction with HSPG on the cell surface, which was taken as an inspiration in this study to engineer antibodies that could block the interaction with HSPG. As the binding between αSyn and HSPG is considered to be dependent on electrostatic interactions, we used a negatively charged peptide that would mimic the negative charges of heparan sulfate (HS) and fused it to the light chain N-terminus of αSyn-specific antibodies. Thus, we expected to achieve an avidity-enhanced binding to αSyn aggregates that may be susceptible to HSPG-mediated uptake into cells. In contrast, we found that the antibody-peptide fusions instead exhibited decreased affinities to αSyn fibrils, which may be due to interference of the peptide with the antibody's binding interface. Additionally, electrostatic repulsion between the peptide and the negatively charged αSyn C-terminus may have impaired antibody binding. Thus, a more fine-tuned approach in mimicking HS properties, including not only its net charge but also charge distribution and sulfation might improve the binding of the antibody-peptide fusion to αSyn fibrils.

Paper IV

Here, we designed αSyn mutants inspired by previous reports on a β-hairpin conformation in the N-terminus of αSyn that has been suggested to occur early during αSyn misfolding. We therefore attempted to promote the formation of this hairpin and stabilize it by introducing cysteines and/or prolines in the αSyn sequence in proximity of the hairpin-forming region. Our results showed very different behaviors of the four different mutants. While two of the mutants still showed a low degree of fibrillation, the other two mutants exclusively formed small oligomers of pentameric or hexameric sizes. All mutants showed a small but significant inhibitory effect on the fibrillation of wt αSyn, increasing the amount of soluble species of αSyn remaining at the end of the aggregation. Thus, our results support the hypothesis that the β-hairpin conformation may play a role in the conversion from oligomers to fibrils. Initial tests to determine the neurotoxicity of the αSyn mutant aggregates showed some effects on the metabolic activity and apoptosis in cell culture, but such small αSyn oligomers are likely to exert different types of effects and at different time frames than large oligomers whose neurotoxicity has been widely studied. Thus, to understand the role of small αSyn oligomers and specifically the β-hairpin in neurotoxicity, testing a broad range of conditions, time frames and parameters is required.

Conclusions and future perspectives

How to target αSyn aggregates?

According to our study on multivalent antibody formats in **Paper I**, avidity-enhanced binding appears beneficial for reducing the antibody's dissociation from the target and for increasing the range of targetable αSyn aggregate sizes. These properties may be beneficial to enhance the overall sensitivity of the antibody for αSyn aggregates perhaps even including smaller sizes that are otherwise not targetable by antibody-based drugs. However, this strategy might not solve the problem that a therapeutic antibody gets captured by abundant but pathologically irrelevant αSyn aggregate species.

Though our approach of targeting αSyn aggregates with a HS-mimicking peptide in **Paper III** was not successful, it may be possible to improve the peptide design in order to better mimic HS characteristics and thus potentially allow to interfere with αSyn cell-to-cell spreading to some degree. However, HSPG-mediated uptake is not the only mechanism by which extracellular αSyn can be taken up into cells. Hence, to interfere with the transmission of αSyn from the extracellular space, one would have to compete with multiple different mechanisms at the same time. Not to mention the transmission mechanisms where αSyn aggregates are transmitted in exosomes or tunneling nanotubes without even entering the extracellular space.

Hence, the small pool of αSyn that is actually available to drugs in the extracellular space, makes it debatable whether any extracellular treatment has a chance against αSyn aggregation and its spreading or if intracellular targeting is the only possibility to interfere with the progression of αSyn pathology.

Overall, there is still a major knowledge-gap regarding the specific characteristics of the pathologically relevant αSyn species which hampers the development of therapeutics specifically targeting those αSyn species that are responsible for neurotoxicity and seeding of aggregation. Our findings in **Paper IV** support the hypothesis of the β-hairpin playing a critical role in the conversion from αSyn oligomers to fibrils. However, it remains to be shown whether the β-hairpin is exclusive to either toxic or seeding-competent αSyn oligomers, if it is a common characteristic of pathological oligomers, or if it is perhaps also present in non-pathological oligomers. Due to their very transient nature, small αSyn oligomers have been missing in investigations on identifying the most pathological αSyn species. Hopefully, stabilized αSyn oligomers as those we presented in **Paper IV** will contribute to a better

understanding of the relevance of small αSyn oligomers and the β-hairpin conformation in αSyn pathology.

How to cross the BBB?

The small proportion of intravenously injected antibodies reaching the brain interstitium makes the need for a strategy of efficient antibody transport across the BBB obvious and is likely one of the reasons why antibodies against αSyn aggregates have not shown efficacy in clinical trials so far. There is currently one approved protein-based drug equipped with a TfR-targeting BBB-shuttle [170] and several more are undergoing clinical trials such as candidates by the companies Roche and Denali showing promising results for the delivery of protein-based drugs to the brain [171,172].

In contrast to the current believe that monovalent TfR-binders are more efficient in crossing the BBB than bivalent binders, we have shown in **Paper II** that likely not the valency but the radius in which RmAb158-scFv8D3 can bind to different TfR on the cell surface determines its transcytosis efficiency. Thus, binding TfR bivalently at a very short distance may not cause the transcytosis-inhibiting effects of TfR-crosslinking as observed with traditional bivalent TfR-binders. However, this hypothesis concluded from *in vitro* experiments remains to be tested *in vivo*.

Besides its expression on the BBB endothelial cells, TfR is also expressed on neurons, microglia and astrocytes which raises the question if TfR-targeting could perhaps even allow protein-drugs to treat intracellular pathologies. Though findings on whether or not TfR-targeting proteins internalize into brain cells are controversial [53,173–175].

Popular science summary

Parkinson's disease is a neurodegenerative disease in which neurons in the brain die. It affects in particular a region in the brain that is responsible for the control of movement, resulting in symptoms such as tremor, muscle stiffness, and balance difficulties. Additionally, the disease can also cause symptoms unrelated to movement such as loss of smell, sleep disturbances, depression, and dementia. Even though the exact reasons for why someone develops Parkinson's disease are not fully understood, a specific protein called alpha-synuclein is thought to play a major role in the disease's progression. Alpha-synuclein is present in neurons in high amounts where it is usually involved in the regulation of signal transmission between neurons. In Parkinson's disease, alpha-synuclein starts to misfold and forms clumps, commonly referred to aggregates, which increase in size and convert into different shapes over time. Some of these aggregates are toxic to neurons as they interfere with various important cellular functions. Others are more potent in promoting healthy alpha-synuclein to misfold or in infecting neighbouring neurons with alpha-synuclein aggregates. Still others may accumulate without causing negative effects to neurons. Thus, removing the pathological alpha-synuclein aggregates from the brain appears to be a promising strategy to treat Parkinson's disease. However, there is currently no drug available that can slow down, stop, or revert the aggregation of alpha-synuclein, which can likely be attributed to that drugs candidates are not specific enough for the pathological forms of alpha-synuclein aggregates. One type of drug that has the potential to overcome these limitations is antibody-based drugs.

Antibodies are proteins which our own immune system produces to protect us from foreign substances that enter our body. Antibodies have a very high specificity and binding strength to their respective targets and a long half-life within the body. These characteristics and our ability to modify antibodies by protein-engineering and produce them in the lab have made them to one of the fastest growing fields in drug development.

The aim of this thesis was to identify and improve strategies to bind pathological alpha-synuclein aggregates by engineering new antibody-based drugs.

In Paper I, we produced two novel antibodies with respectively four and six binding sites for alpha-synuclein. In comparison to natural antibodies, which contain only two binding sites for their target, these novel antibodies were able to bind stronger to alpha-synuclein aggregates and have the

potential to also bind smaller aggregates which have been suggested to be the more toxic form of alpha-synuclein aggregates.

One mechanism of how alpha-synuclein aggregates are thought to spread between cells is through their interaction with heparan sulfate proteoglycans (HSPG), which are carbohydrate-modified proteins on cell surfaces. This interaction has been suggested to be based on the positive charges that cluster on alpha-synuclein aggregates binding to the negative charge of HSPGs. In Paper III, we attempted to mimic this interaction by equipping alpha-synuclein aggregate-binding antibodies with a short negatively-charged protein tail to enhance their binding strength to the aggregates. However, we found that those novel antibodies bound weaker to alpha-synuclein aggregates than antibodies without the tail, which can likely be ascribed to the protein tail blocking the antibody's binding sites or through electrostatic repulsion from the alpha-synuclein aggregates.

In Paper IV, we designed novel alpha-synuclein mutants with the aim to produce aggregates that are stabilized in a particularly small aggregate form. Such small alpha-synuclein aggregates appear very early during the disease and are potentially highly pathological but are difficult to study due to their instability. We have shown that two of our novel alpha-synuclein mutants were indeed unable to convert into larger aggregates but instead were stabilized as aggregates of five to six subunits.

In Paper II, we focused on the challenge of bringing antibody-based drugs into the brain. The transport of substances from the blood into the brain is tightly regulated by the blood-brain barrier (BBB) formed by the capillaries that run through the brain. Thus, only a very small proportion of an intravenously injected dose of antibodies reaches the brain. However, antibodies can be engineered to hitchhike on natural transport proteins across the BBB. One such protein is the transferrin receptor (TfR), which naturally transports iron across the BBB into the brain. It has previously been shown that antibodies that bind TfR with two arms can cluster several TfR on the BBB surface, which reduces their transport efficiency compared to antibodies with only one TfR-binding arm. In contrast to those observations, we have shown in Paper II that engineering an antibody equipped with two TfR-binding arms in a way that drastically decreases the distance between those two arms helps this antibody to be transported equally well across an *in vitro* BBB model as an antibody with only one TfR-binding arm.

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