Development of new affinity proteins for neurodegenerative disorders

LINNEA CHARLOTTA HJELM

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Academic Dissertation which, with due permission of the KTH Royal Institute of Technology, is submitted for public defence for the Degree of Doctor of Philosophy on Friday the 24th March 2023, at 10:00. in F3, Lindstedtsvägen 26, Stockholm.

Doctoral Thesis in Biotechnology
KTH Royal Institute of Technology
Stockholm, Sweden 2023
People who wonder if the glass is half empty or full miss the point. The glass is refillable.

-Unknown-
Quoted of patient with dementia
Neurodegenerative disorders include a full spectrum of diagnoses, including dementias and other neuronal diseases, characterised by degradation of neurons in the brain occurring along with disease progression. Amongst the dementias, the most prevalent are Alzheimer’s (AD) and Parkinson’s disease (PD) that affect millions of people worldwide. During the last years, advancements in potential treatments have been made where the first two clinical antibodies have been approved by the US Food and Drug Administration (FDA) for a disease modifying effect on Alzheimer’s disease.

As alternatives to antibodies, other types of affinity reagents that are based on non-immunoglobulin protein scaffolds are also investigated. Such alternative scaffolds often demonstrate distinct and complementary properties compared to antibodies. In this thesis, the development of a new type of affinity protein scaffold called sequestrin is described. Sequestrins are derived from the affibody molecule and comprise two heterogenic subunits with truncated N-terminals fused as a head-to-tail construct. Sequestrins undergo a structural rearrangement upon target binding and forms a stable complex. The scaffold is designed for interactions with disease-related amyloidogenic peptides e.g. amyloid beta and alpha-synuclein involved in AD and PD, respectively. In the first paper, a sequestrin library was developed and its compatibility with phage display was investigated. Successful panning against the amyloid beta peptide resulted in binders with high affinity. Further on in paper II, the alpha-synuclein peptide was targeted and sequestrins with low nanomolar affinities were obtained. All sequestrins displayed structural rearrangement upon target engagement, which stabilized the interaction to the target peptides and further inhibited toxic aggregation, opening up for future studies of disease modifying effects in vivo.

When targeting the brain, passage through the blood–brain barrier (BBB) is an obstacle that needs to be addressed to reach sufficiently high therapeutic concentrations. To overcome this barrier, brain shuttles have been developed with the capability to transport a cargo over the BBB. One such mechanism of transportation is by receptor-mediated transcytosis, which is utilized by e.g. the transferrin receptor (TfR). In paper III, a TfR-targeting shuttle was investigated for BBB passage when fused to a sequestrin targeting the amyloid ABSTRACT

Neurodegenerative disorders include a full spectrum of diagnoses, including dementias and other neuronal diseases, characterised by degradation of neurons in the brain occurring along with disease progression. Amongst the dementias, the most prevalent are Alzheimer’s (AD) and Parkinson’s disease (PD) that affect millions of people worldwide. During the last years, advancements in potential treatments have been made where the first two clinical antibodies have been approved by the US Food and Drug Administration (FDA) for a disease modifying effect on Alzheimer’s disease.

As alternatives to antibodies, other types of affinity reagents that are based on non-immunoglobulin protein scaffolds are also investigated. Such alternative scaffolds often demonstrate distinct and complementary properties compared to antibodies. In this thesis, the development of a new type of affinity protein scaffold called sequestrin is described. Sequestrins are derived from the affibody molecule and comprise two heterogenic subunits with truncated N-terminals fused as a head-to-tail construct. Sequestrins undergo a structural rearrangement upon target binding and forms a stable complex. The scaffold is designed for interactions with disease-related amyloidogenic peptides e.g. amyloid beta and alpha-synuclein involved in AD and PD, respectively. In the first paper, a sequestrin library was developed and its compatibility with phage display was investigated. Successful panning against the amyloid beta peptide resulted in binders with high affinity. Further on in paper II, the alpha-synuclein peptide was targeted and sequestrins with low nanomolar affinities were obtained. All sequestrins displayed structural rearrangement upon target engagement, which stabilized the interaction to the target peptides and further inhibited toxic aggregation, opening up for future studies of disease modifying effects in vivo.

When targeting the brain, passage through the blood–brain barrier (BBB) is an obstacle that needs to be addressed to reach sufficiently high therapeutic concentrations. To overcome this barrier, brain shuttles have been developed with the capability to transport a cargo over the BBB. One such mechanism of transportation is by receptor-mediated transcytosis, which is utilized by e.g. the transferrin receptor (TfR). In paper III, a TfR-targeting shuttle was investigated for BBB passage when fused to a sequestrin targeting the amyloid
beta peptide, resulting in a higher penetration through the BBB, and maintained functionality of the sequestrin.

High-throughput \textit{in vitro} methods would facilitate development of novel brain shuttles. Thus, in paper IV, a transwell system based on nanofibrillar silk membranes with murine brain endothelial cells was developed. Evaluation of the method using a TfR-specific antibody demonstrated higher transfer over the barrier compared to an isotype control and the method has potential to facilitate screening of transcytosis capability of brain shuttles.

In paper V, TfR-specific affibody-based brain shuttles were developed and investigated for transcytosis capability using the \textit{in vitro} transcytosis assay. A panel of affibody molecules were evaluated, demonstrating both cross-species reactivity to murine and human TfR and active receptor-mediated transcytosis. These candidates could thus potentially be used in further development of CNS-targeting therapeutics.

In conclusion, a new sequestrin scaffold was developed that can be utilised for targeting amyloidogenic peptides found in neurodegenerative disorders. An affibody-based brain shuttle was also developed, which showed transcytosis capability. In the future, the new brain shuttle might be combined with sequestrins to create multifunctional fusion proteins for facilitated delivery over the BBB, which hopefully can result in therapeutic concentrations in the brain even when administered with a lower dosage.

\textbf{Keywords}: Protein engineering, sequestrins, amyloid beta, alpha synuclein, phage display, affibodies, blood–brain barrier, Transferrin receptor, receptor-mediated transcytosis, recombinant spider silk
Neurodegenerativa sjukdomar är en samlingsterm för olika tillstånd som bland annat inkluderar demenssjukdomar som Alzheimers och Parkinsons sjukdom, där hjärnans neuroner degraderas med sjukdomsutvecklingen. Miljontals personer är drabbade av dessa sjukdomar och tyvärr finns det få tillgängliga behandlingar. Nyligen har två antikroppar godkänts av amerikanska läkemedelsverket (the US Food and Drug Administration, FDA) som läkemedel för behandling av Alzheimers sjukdom, och dessa två är de första i sitt slag som påverkar sjukdomsförloppet.


en tidigare validerad TfR-transportör urskiljas från negativa kontrollen i denna metod.

I artikel V beskrivs arbetet med att utveckla ett antal varianter av affibodies med målet att fungera som transportörer via TfR för transport in till hjärnan. Dessa affibodies karakteriserades för sin funktion att binda till både humant och murint cellulärt uttryckt TfR. Vidare visades det att även andra generationens affibodies för TfR kunde genomgå transcytos över barriärmodellen.

Sammanfattningsvis presenteras det i denna avhandling en utveckling av sequestrinstrukturen och framtagande av nya bindare för inhibering av aggregationsprocessen hos proteiner som frekvent återfinns inom neurodegenerativa sjukdomar, samt utvecklingen utav affibody-transportörer över blod–hjärnbarriären.

**Nyckelord:** proteinvetenskap, sequestriner, amyloid beta, alfa-synuklein, fag-display, affibodies, blod–hjärnbarriären, transferrinreceptorn, receptor-medierad transcytos, rekombinant spindelsilke

De nyligen godkända läkemedlen är biologiska läkemedel i form av proteiner, vilket är mycket större strukturer än t ex. småmolekylen paracetamol som finns i Alvedon. Proteiner är uppbyggda av en följd av aminosyror. De återfinns i många olika former och har många olika funktioner i kroppen, bland annat för att spjälka mat, eller bekämpa en infektion. Kroppen kan skapa antikroppar mot en infektion som är specifika mot just den infektionen och varianten som man har insjuknat i. Detta gör antikropparna väldigt effektiva, men de kan har sämre effektivitet mot till exempel en ny variant av viruset jämfört med den som har infekterat kroppen tidigare, vilket gör att man kan bli sjuk igen och behöver upprepa processen. Kroppen är duktig på denna typ av upprepning och kan hitta nya varianter och kombinationer av aminosyrorna i antikroppssekvensen för att skapa nya antikroppar mot nya infektioner, eller för att förbättra redan befintliga antikroppar mot en ny variant av en infektion. Processen kan likaså visas vid artevolution och är ett kraftfullt verktyg även i en laboratoriemiljö.

Användning utav evolutionsprocessen har varit central i denna avhandling för utveckling och selektion av sequestriner och affibodymolekyler mot olika målproteiner. Dessa två proteinstrukturer är betydligt mindre i storlek än konventionella antikroppar. De har definierade delar av sin aminosyrasekvens som är lämpliga att modifera när man vill hitta varianter som binder till nya målproteiner. Genom att kontrollerat variera en eller flera olika aminosyror kan man skapa milliontals olika varianter, det vill säga ett bibliotek av


Att väja rätt protein från dessa milliontals varianter är inte lätt, men med bättre metoder och design av bibliotek ökar sannolikheten att hitta en variant som är lämplig att fortsätta utveckla och studera. Forskningen som ligger till grund för den här avhandlingen har förhoppningsvis bidragit till att komma ett steg närmre en ny läkemedelskandidat.
LIST OF APPENDED PAPERS AND MANUSCRIPTS

I. “Construction and Validation of a New Naïve Sequestrin Library for Directed Evolution of Binders against Aggregation-Prone Peptides”
Authors: Linnea C. Hjelm, Hanna Lindberg, Stefan Ståhl, and John Löfblom
doi: 10.3390/ijms24010836

II. “Sequestrins inhibiting amyloidogenic aggregation-prone peptides”
Authors: Linnea C. Hjelm, Hanna Lindberg, Wojciech Paslawski, Per Svenningsson, Stefan Ståhl, and John Löfblom
Manuscript

III. “An Affibody molecule is Actively Transported into the Cerebrospinal Fluid via Binding to the Transferrin Receptor”
Authors: Sebastian W. Meister, Linnea C. Hjelm, Melanie Dannemeyer, Hanna Tegel, Hanna Lindberg, Stefan Ståhl, and John Löfblom
doi: 10.3390/ijms21082999

IV. “In vitro Blood–Brain Barrier Model based on Recombinant Spider Silk Protein Nanomembranes for Evaluation of Transcytosis capability of biomolecules”
Authors: Linnea C. Hjelm, My Hedhammar and John Löfblom
Submitted to Biotechnology Journal 2023-01-13

V. “Affibody molecules intended for receptor mediated transcytosis utilising the transferrin receptor for brain delivery”
Authors: Linnea C. Hjelm, Hanna Lindberg, Stefan Ståhl, and John Löfblom
Manuscript
**RESPONDENT’S CONTRIBUTION TO APPENDED PAPERS AND MANUSCRIPTS**

I. Conceptualization; methodology; software; validation; formal analysis; investigation; data curation; writing—original draft preparation; writing—review and editing; visualization.

II. Conceptualization; methodology; software; validation; formal analysis; investigation; data curation; writing—original draft preparation; writing—review and editing; visualization.

III. Investigation, and writing—review and editing together with co-authors.

IV. Conceptualization; methodology; software; validation; formal analysis; investigation; data curation; project administration; writing—original draft preparation; writing—review and editing; visualization.

V. Conceptualization; methodology; software; validation; formal analysis; investigation; data curation; writing—original draft preparation; writing—review and editing; visualization.
PAPERS NOT INCLUDED IN THE THESIS


PUBLIC DEFENSE OF DISSERTATION

This thesis will be defended Friday the 24th of March 2023 at 10:00, F3, Lindstedtvägen 26, Stockholm, for the degree of “Teknologie doktor” (Doctor of Philosophy, Ph.D.) in Biotechnology.

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## Abbreviations

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<th>Description</th>
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<tr>
<td>Aa</td>
<td>Amino acid</td>
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<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<tr>
<td>ABD</td>
<td>Albumin binding domain</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>ADA</td>
<td>Anti-drug antibody</td>
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<td>AMP</td>
<td>Antimicrobial peptide</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>aSN</td>
<td>α-synuclein</td>
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<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
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<tr>
<td>BAD</td>
<td>Beta amyloid dysfunction</td>
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<tr>
<td>BBB</td>
<td>Blood–brain barrier</td>
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<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CDR</td>
<td>Complementarity-determining region</td>
</tr>
<tr>
<td>CDR–SB</td>
<td>Clinical Dementia Rating Scale – Sum of Boxes</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DIAN-TU</td>
<td>Dominantly Inherited Alzheimer Network Trials Unit</td>
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<td>DLB</td>
<td>Dementia with Lewy bodies</td>
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<td>DMT</td>
<td>Disease modifying treatment</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EMA</td>
<td>European Medicines Agency</td>
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<td>EMG</td>
<td>Electromyography</td>
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<td>EOAD</td>
<td>Early-onset Alzheimer’s disease</td>
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<tr>
<td>epPCR</td>
<td>Error-prone polymerase chain reaction</td>
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<tr>
<td>Fab</td>
<td>Fragment antigen binding</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
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<td>FcRn</td>
<td>Neonatal Fc receptor</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>H-Ft</td>
<td>Heavy chain ferritin</td>
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<td>HSA</td>
<td>Human serum albumin</td>
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<td>Hsp</td>
<td>Heat-shock proteins</td>
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<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IGFR</td>
<td>Insulin-like growth factor 1 receptor</td>
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<tr>
<td>ISF</td>
<td>Interstitial fluid</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
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<tr>
<td>$K_a$</td>
<td>Association rate constant</td>
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<tr>
<td>$K_d$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$K_{D}$</td>
<td>Equilibrium dissociation constant</td>
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<tr>
<td>LOAD</td>
<td>Late-onset Alzheimer’s disease</td>
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<tr>
<td>LOF</td>
<td>Loss of function</td>
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<tr>
<td>LRP1</td>
<td>Low-density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
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<tr>
<td>MOA</td>
<td>Mode of action</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<td>MSA</td>
<td>Multiple system atrophy</td>
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<tr>
<td>NCS</td>
<td>Nerve conduction study</td>
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<tr>
<td>NDs</td>
<td>Neurodegenerative disorders</td>
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<td>NFTs</td>
<td>Neurofibrillary tangles</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NVU</td>
<td>Neurovascular unit</td>
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<tr>
<td>$p_{app}$</td>
<td>Apparent permeability</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>RMT</td>
<td>Receptor-mediated transcytosis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>scFab</td>
<td>Single-chain Fab</td>
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<tr>
<td>scFv</td>
<td>Single-chain fragment variable</td>
</tr>
<tr>
<td>sdAb</td>
<td>Single-domain antibody</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>Sq</td>
<td>Sequestrin</td>
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<tr>
<td>TC</td>
<td>Tissue culture</td>
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<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin Receptor</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
<td>$T_m$</td>
<td>Thermal melting</td>
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<tr>
<td>TV</td>
<td>Transport vehicle</td>
</tr>
<tr>
<td>VNAR</td>
<td>Shark variable domain of new antigen receptor</td>
</tr>
<tr>
<td>Z</td>
<td>Affibody</td>
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Acknowledgements

References
CHAPTER 1
NEURODEGENERATIVE DISORDERS

Dementia includes a whole spectrum of different diagnoses such as, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, frontotemporal dementia, and vascular dementia among several others.[1] For many of these dementias the common denominator is the degeneration, or breakdown, of the neuronal pathways in the brain yielding symptoms as memory or language loss, change of personality, and impairment of motor functions.[2] Neurodegenerative disorders (NDs) is a term that is used to distinguish dementias and other disorders that cause neurodegeneration to the brain. In this thesis, the emphasis is on Alzheimer’s disease and Parkinson’s disease.

In 2019, dementia affected 57.4 million people worldwide with a prevalence of 0.69%.[2, 3] In 2050, the number of people with a dementia diagnosis is expected to increase with over 300% to over 152.8 million cases globally.[2] The increase of dementia cases is linked to the growth of the global population and life expectancy, which can be seen as a heterogenetic disposition of cases in the world with a female–male ratio of 1.69 ± 0.05. In the high-income Asia Pacific region and Western Europe, the number of cases is expected to increase 53–74% in the next 30 years due to increased life expectancy of the population. In contrast, the prevalence in Africa and the Middle East is expected to increase 357–367% during the same period primarily due to population growth.[2]

The cost of care for dementia patients was in 2015 estimated to $818 billion.[3] To be able to handle this increasing number of patients and cost, multifaceted approaches are needed where efficient treatments, accurate diagnostic methods, good public healthcare, support from the society, and elderly care are essential to cope with the challenge.[2]

This thesis aims to give an introduction to the NDs, with focus on Alzheimer’s and Parkinson’s disease, and the protective blood–brain barrier. The molecular mechanisms of the aggregation-prone proteins associated to the diseases, and the general theories for disease development is further explained as they are central to the development of the sequestrin scaffold covered in
Neurodegenerative disorders

paper I and II. Sequestrins are developed with the aim to generate a therapeutic effect by inhibition of the aggregation process via encapsulation of the target protein and is further discussed herein. As the therapeutic targets are located in the brain, delivery into this protected environment is an important consideration. Thus, this thesis also includes a general background of the protective blood–brain barrier and current strategies for transportation across it, primarily through a Trojan horse approach. In paper III–V, utilisation and developments of a new Trojan horse strategy are investigated together with the development of a transcytosis screening model, which aims to ease the development process for new Trojan horse proteins.

1.1. What are Neurodegenerative Disorders

Neurodegenerative disorders are classified as disorders where the neurons of the brain are gradually losing their functionality and decreasing in numbers. Most NDs are associated with pathological protein deposits as a result of misfolding and aggregation of certain proteins. Both proteins and the aggregation process can vary between the disorders. The causes and pathways leading to neurodegeneration are suggested to be several and include amongst others, mitochondrial dysfunction, alerted energy metabolism, oxidative stress, microglial activation, DNA damage and mutations, disruption of cellular and axonal transportation, and neuroinflammation. These pathways are linked to some or several NDs, but are likely not the only pathways leading to the dysfunction and death of neurons. Therefore, a fundamental understanding of the pathogenic pathways and the complex interconnection between them is essential for developing therapeutics for the diseases. Unfortunately, we are not there yet.

The brain is a highly interconnected network of neurons that controls both our active and autonomic impulses. Different areas in the brain host different functionalities (figure 1.1). The frontal lobe is associated with thinking, planning, emotions, behaviour, and personality. The temporal lobe is associated with memory understanding language and expressed behaviour. Autonomic functions and cerebellum coordination, and voluntary movements are controlled by the brain stem. The occipital lobe interprets vision inputs, and the parietal lobe is associated to perception, spelling, sensory input, and body orientation. Thus, when neurons get damaged or are subjected to a toxic environment, it can affect a wide range of the brain functionality depending on where the damage occurs. Luckily, the brain is highly adaptable and even if some neurons become damaged a signal pathway can be redirected and still be functional, due to the budding and pruning capabilities of neurons. This is something that the brain is exceptionally good at early in life and is part of an
essential process for the development of the brain. As we get older, this process is slowing down, and regeneration or redirection of these pathways become harder. Eventually the neuronal loss will lead to permanent damage and a general shrinkage of the brain. Depending on the disease, different parts of the brain are affected, leading to the variety of symptoms seen in NDs (figure 1.1).[4],[5]

![Diagram showing brain regions associated with Neurodegenerative Disorders](image)

**Figure 1.1.** The brain regions (colour from left to right) with associated NDs area of degeneration. Some NDs are associated with more than one region. The main molecular pathology is listed and can be found in several of the NDs.

In NDs the brain degenerates as the disease progresses, and up to 20% of the initial brain matter volume can be lost. This is usually seen for patients with dementias and NDs when the progression is severe. Since much of the functionality is lost at this stage, treatment is therefore challenging. Thus, a prophylactic treatment and early diagnosis is key aspects for efficient treatment options. In for example Alzheimer’s disease, the accumulation of amyloid beta (Aβ) tend to start as early as 10–15 years before the symptoms of memory loss appear (figure 1.2). The adaptable neuronal network delays the symptoms until the damage becomes too severe, however with the extensive loss of neurons, a decline of the cognitive function will be inevitable.[6]

The blood brings essential oxygen and nutrients to the brain. Neurons are, however, sensitive to their environment and can die if it is changed significantly. The blood–brain barrier (BBB) contributes to maintaining a stable environment and protects the brain from the variability of different
components in the blood (chapter 2). With this barrier, the brain environment is maintained and separated from the rest of the body. Changes that occur within the brain environment will however affect the neurons, which can lead to loss of functionality or death. The changes depend on the type of ND, but they communally yield a change in the brain homeostasis, which can have its basis in protein misfolding, mutation, and of loss of function.

Figure 1.2. Development of Alzheimer’s disease over a few different stages, from healthy, preclinical, mild cognitive impairment (MCI), and dementia. The lines show the progression and development of different key points such as, amyloid beta (Aβ) accumulation, synaptic dysfunction, tau mediated injury, loss of brain structure, cognitive impairment, and clinical function. Marked is the threshold of detection, even if diagnosis by today’s methods is done late in the disease progression. With high or low risk of AD by mutations and environmental factors the cognitive onset can vary in time. Figure drawn with inspiration from Sperling et al. [6]

1.2. Proteins, homoeostasis, and aggregation

The deoxyribonucleic acid (DNA) found in cells encodes for all the proteins in the human body. The central dogma is the process from where sections of the DNA are transcribed into ribonucleic acid (RNA) and translated by ribosomes into a chain of amino acids that typically assemble into a three-dimensional form of a protein. Thus, by changing one letter in the DNA code, an amino acid in the protein can be altered, which might cause the protein to become non-functional. Some changes in the DNA are commonly found in NDs and are referred to as disease mutations, which can create pathological protein variants specific for the disease. These mutations can sometimes be specific to a certain population or regional area as in e.g. Alzheimer’s disease where there are several mutational variants including the Swedish mutation, the Artic mutation, the London mutation, or the Flemish mutation. All these mutations,
cause an effect on the protein homoeostasis in the brain, which is yielding an increased risk of developing the disease.\cite{7,8}

When the translation process occurs, the protein often folds in a certain order to become functional. To help in the folding, cells have chaperones, directing the process. The folding is driven by both entropy and enthalpy, and chaperones catalyse folding of the protein into certain structural elements. However, in some cases the folding fails. When this happens, the malfunctioning protein is usually broken down by the cell ubiquitination protein degradation pathway or by autophagy, and the amino acids reused for new proteins. This breakdown and build-up of proteins are called protein homeostasis, and the cell holds a functional equilibrium between the two (figure 1.3A).\cite{9} However, in some cases, an alternative energy-favourable structure other than the functional one exists, driving a structural shift of the protein, which can lead to aggregation. Since these aggregates are often energetically favourable, they might be difficult for the cell to break down. By time, more and more will fall into this energy state and thereby large aggregate deposits can be formed and become a permanent addition to the intracellular or extracellular environment (figure 1.3B). Protein deposits like this can induce toxic effects leading to cell death and disease, which is seen in several of the NDs. Recognition of the protein conformation is central in development of therapeutic proteins as further covered in section 1.4.3.

1.3. Different types of neurodegenerative disorders

There are several different NDs, which include, Alzheimer’s disease (AD), Parkinson’s disease (PD), frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), Parkinson’s disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), Huntington’s disease (HD), and others.\cite{10} The prevalence, time of onset for the disease, pathogenesis and mutations, and progression of the disease varies between the disorders. What the different NDs have in common is the aggregation of proteins within the brain (figure 1.1, table 1.1), which can be symptomatic or disease causing depending on the ND (figure 1.4), the pathway or pathways and correlations are still investigated. The work in this thesis revolves around AD and PD.
Figure 1.3. A) Protein homoeostasis with biogenesis build-up of proteins with intermediate folding before attaining the correct structure. Protein breakdown occurs by ubiquitination (ub) or autophagy of aggregates. The degraded protein can be re-used by the cell as amino acids for new proteins. Misfolding events occur in the cell frequently, by the help of e.g. disaggregates the aggregation can be reversible. The figure is created with inspiration from Sala et al.\textsuperscript{[9]}. B) The free energy is minimised by folding events of the protein and can be catalysed to the correct pathway by chaperones. By off-pathway events, aggregates and oligomers might form, which have a lower
free energy state than the native state. With the lower energy, this would be a preferred protein folding but is directed by the chaperone presence into the native state. The Aβ pathway of aggregation, where soluble monomer attains a β-hairpin structure in solution. The aggregation process is initiated by the structural change and occurs in the order of, oligomerisation, protofibril and fibril formation. It is believed that oligomeric and protofibril products may be reversible.

Figure 1.4. Related genetic causes, proteinopathies, and disease correlations for different subtypes and NDs. The correlation implication on a specific subtype is weighted by the thickness of the line associated to an arrow. The molecular pathology is observed in several different subtypes and types of NDs. The molecular pathology can itself be divided in several subtypes as TDP-43 type A, type B, and type C, which is not specified in the figure, but correlates further to certain subtypes in the clinical phenotype. Drawn with inspiration from Pievani et al.[11].

1.4. Alzheimer’s disease

Alzheimer’s disease (AD) is the most common dementia and has a global prevalence that increases with age.[2] Approximately 3.3% of people older than 65 years are estimated to suffer from the disease (table 1.1).[2, 3] As it has a relatively late onset (around 60 years of age) compared to other NDs, the prevalence is linked to the age of the population, and thus more common in high-income countries.[2] AD can be divided into inherited disease (familial) and sporadic cases, of which sporadic stands for 90–95% of all AD cases. The different types have different correlations to the disease, where familial AD is mutation-linked[7] and sporadic cases have a vast number of associations such as, environmental, DNA methylation levels, and genetic mutations.[12] AD is
### Table 1.1: Disease information about some of the higher prevalence neurodegenerative disorders. Time for onset, disease pathogenesis and progress is listed. Alzheimer’s disease has the highest prevalence among the diseases. Genetic causes and proteinopathies are further shown in figure 1.4.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Global prevalence</th>
<th>Onset (years)</th>
<th>Life expectancy (years)</th>
<th>Affected brain regions</th>
<th>Pathology</th>
<th>Deposits</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>5/1,000</td>
<td>EOAD: 30–mid 60s, LOAD: 60+</td>
<td>8–10</td>
<td>Cerebral cortex, hippocampus</td>
<td>Aβ, Tau</td>
<td>Extracellular plaques, intracellular tangles</td>
<td>PET, SPECT (Aβ, tau)</td>
</tr>
<tr>
<td>Parkinson’s disease (PD)</td>
<td>1/5,000</td>
<td>60+</td>
<td>10–20</td>
<td>Substantial nigra</td>
<td>α-synuclein</td>
<td>Intracellular cytoplasmic inclusions</td>
<td>Neurological and physical abilities, PET of dopamine</td>
</tr>
<tr>
<td>Frontotemporal dementia (FTD)</td>
<td>3/100,000</td>
<td>40–65</td>
<td>6–8</td>
<td>Frontal and temporal lobe</td>
<td>TDP-43, FUS, Tau</td>
<td>Tau, TDP-43 or FUS accumulations</td>
<td>Mental abilities</td>
</tr>
<tr>
<td>Dementia with Lewy bodies (DLB)</td>
<td>6/100,000</td>
<td>50+</td>
<td>5–7</td>
<td>Cerebral cortex, limbic cortex, hippocampus</td>
<td>α-synuclein</td>
<td>Levy bodies, intracytoplasmic neurons</td>
<td>Mental abilities</td>
</tr>
<tr>
<td>Multiple system atrophy (MSA)</td>
<td>4/100,000</td>
<td>50–60</td>
<td>7–10</td>
<td>Cerebellum</td>
<td>α-synuclein</td>
<td>Glia cytoplasmic inclusions in cytoplasm of oligodendrocytes</td>
<td>PET, MRI, dopamine as exclusion methods</td>
</tr>
<tr>
<td>Huntington’s disease (HD)</td>
<td>6/1,000,000</td>
<td>30–50</td>
<td>10–30</td>
<td>Striatum, cerebral cortex</td>
<td>Huntingtin</td>
<td>Intracellular nuclear inclusions</td>
<td>Genetic test</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>2/100,000</td>
<td>40–mid 60s</td>
<td>2–5</td>
<td>Spinal cord, motor cortex, brain stem</td>
<td>TDP-43, SOD1, FUS</td>
<td>Intraneuronal aggregates</td>
<td>MRI, EMG, NCS, blood test</td>
</tr>
<tr>
<td>Progressive supranuclear palsy (PSP)</td>
<td>11/100,000</td>
<td>45–75</td>
<td>5–7</td>
<td>Brain stem</td>
<td>Tau</td>
<td>Tau inclusions in intracytoplasmic neurons, astrocytes, oligodendrocytes</td>
<td>MRI, brain volume</td>
</tr>
</tbody>
</table>
generally believed to be correlated with the aggregation of the Aβ peptide (figure 1.3C) and it has been the main target in therapeutic development during several years. The pathway for induced toxicity and mode of action (MOA) is still debated. The generally considered theories are described below.

1.4.1. What happens in Alzheimer’s disease

The amyloid precursor protein (APP) family consists of three different forms, the APP and APP-like protein 2 (APLP2) that are expressed in the whole body, and APLP1 that is only expressed in the brain. APP, that within the brain is predominantly expressed on neurons, is the only form in the family that has an Aβ peptide domain and the Aβ peptide is thus a cleavage product of APP. The large ectodomain of APP family members binds to e.g., heparin, zinc, and copper that can cause formation of reactive oxygen spices (ROS). The APP thus aids in protecting the neuronal environment by limiting the development of ROS. The APP is well trafficked within the cell and undergoes compartmentalised cleavage by different secretases (figure 1.5, 1.6). The most abundant β-secretase BACE1 fuses with APP in early endosomes and by endosome maturation and acidification cleaves the protein. Further processing by the γ-secretase is speculated to occur in the late endosome or in the Trans Golgi Network which might influence the cleavage position. The cleavage products are either degraded in the lysosome or secreted out from the neuron by transport vesicles or recycling endosomes and by iron export from the cell. APP can be processed in two different pathways, the non-amyloidogenic or the amyloidogenic pathway (figure 1.5). More information on the domains, functions and importance of the APP family can be found elsewhere.

Neuronal activity is defined by the electric activity in the cell. A high activity is correlated to an increased APP cleavage by the non-amyloidogenic pathway by α-secretase (figure 1.5). Neuronal synapses that have a high activity also have an increased density of APP, which is speculated to be under an active feedback loop. This neuronal activity is also related to increased amounts of neurotransmitters. With the α-secretase cleavage, the APP is cleaved into sAPPα and membrane bound CTFα. The membrane bound CTFα can further be cleaved at the membrane-cytosol interface on the membrane side by γ-secretase to ACID and P3. The function of P3 is likely dual. At certain amounts, P3 has a neuroprotective function, but when overexpressed, as in Downs syndrome, it has a toxic effect. The ACID product, is internalised and translocated into the nucleolus where it works as a regulatory element for transcription of genes mainly related to induction of apoptosis.
In the amyloidogenic pathway, APP is cleaved by the abundant $\beta$-secretase creating sAPP$\beta$ and membrane bound CTF$\beta$ (figure 1.5). This can further be cleaved by $\gamma$-secretase and release the A$\beta$ fragment in different isoforms that are between 36 and 49 amino acids (aa) long,\cite{7,14} where about 90% are of the length 40 amino acids.\cite{7,14} Both the sAPP$\alpha$ and sAPP$\beta$ are released to the extracellular environment, where they are involved in neurite outgrowth by interaction to the p75 neurotrophin receptor. The sAPP$\alpha$ fragment is further involved in processes such as cellular growth, synapse formation, and reduction of spine density. The sAPP$\beta$ function is not fully understood but has been suggested to be involved in the process of pruning spines and being involved in axonal death, both as an inhibitor and progenitor. A complete loss of the APP protein leads in knock-out mice to spatial memory deficits (navigation) and a reduced number of synapses, which is reducing the brain functionality, suggesting its importance.\cite{7,14}

**Figure 1.5.** The cleavage pathway for APP, including the non-amyloidogenic and amyloidogenic pathways which generates soluble sAPP$\alpha$ and P3 or sAPP$\beta$ and A$\beta$. The ACID fragment is created regardless of the pathway. The cleavage takes place on the extracellular side of the membrane in the primary step, and $\gamma$-secretase cleaves within the cell membrane as further specified in figure 1.6. Figure is drawn with inspiration from Chen et al.\cite{14}
Neurodegenerative disorders

The cleavage position of γ-secretase on APP differs and generates intrinsically unstructured Aβ fragment of different lengths (figure 1.6).[7] However, in solutions of physiological conditions, Aβ can form a distinct structure depending on the cleavage site.[14] In the peptides Aβ_{1-40} and Aβ_{1-42}, the first 14 amino acids are unstructured, whereas amino acid 15–36 forms an alpha helical structure with a kink or hinge at position 25–27 (figure 1.6B, C). The C-terminal shape differs between the isotypes. The two additional hydrophobic amino acids added to the C-terminus in the 1–42 isotype switches the equilibrium from an α-helix to a β-hairpin, formed with amino acids 31–34 and 38–41 (figure 1.6D). This reduces the flexibility of the C-terminus and induces a higher probability for aggregation (figure 1.6.E).[14]

Even if the pathological hallmarks of Aβ as protein deposits are linked to AD progression, Aβ monomers has a vital biological function. Monomeric Aβ is either secreted after cleavage or become linked in lipid rafts as soluble or aggregated form on the cell membrane.[7] The soluble Aβ can interact with secreted proteins as for example, Apolipoprotein E (ApoE) and several cellular receptors, or be endocytosed by nearby cells. This unspecific interaction with receptors might trigger undesired pathways causing neuronal stress or toxicity or microglia activation, and some examples of interacting receptors are covered by Chen et al.[14] Excess Aβ levels in the neurons have a toxic effect on mitochondria and absence of Aβ monomers, or too low levels in the synapse, impairs the neuronal function.[14],[15] Thereby, it has been suggested that Aβ_{1–42} is neuroprotective, to a certain degree.[16] Soluble Aβ is further passed over the BBB by either carriers as ApoE or receptors as low-density lipoprotein receptor-related protein 1 (LRP1), which regulates the concentration in the brain. Aβ monomers and oligomers functions as an antimicrobial peptide (AMP) both in blood and in cerebrospinal fluid (CSF) and have shown to be protective of infections.[17],[18] Thus, the levels of monomeric Aβ in the brain are regulated by several pathways such as, by the Aβ-type and cleavage, BBB and neuronal homeostasis, vascular clearance, and degradation.

1.4.2. Theories behind Alzheimer’s disease

Mutations in 18 amino acids of the 770 amino acids long APP gene are correlated to familial Alzheimer’s disease (FAD) (figure 1.6A). However, over 30 mutations correlate with sporadic cases, where the same position can be mutated to different amino acids, such as D678H or D678N, and cause disease. So far only the A673T mutation in APP is known to be protective in AD and is found at high prevalence. Most mutations correlated to FAD are close to the different secretase cleavage sites and might therefore affect the trafficking,
signal pathways, and proteolytic cleavage of APP. The London mutation (V717I) and the Flemish mutation (A692G) can direct the γ-secretase cleavage site towards Aβ₁₋₄₂. The Swedish mutation (K670N, M671NL) has been shown to make the APP a better BACE-1 (β-secretase) substrate and thus increases cleavage by the amyloidogenic pathway (figure 1.6A). Duplication of the APP gene and increased APP levels, has been shown to affect downstream processes such as axonal transportation and endosomal formation in the neurons. Thus, toxic effects might not only be from the APP cleavage, but also by disruption of other cellular processes which the neurons are highly sensitive to. Since the disease correlation between APP and AD is not fully understood, several other hypotheses are also investigated including the amyloidogenic cascade hypothesis, Aβ oligomer hypothesis, the beta amyloid dysfunction hypothesis, cholinergic hypothesis, tau hypothesis, blood-brain barrier (BBB) homeostasis of Aβ hypothesis, diabetes type III hypothesis, ApoE hypothesis, and effects by environmental implications. In this thesis, the work is based on Aβ-related hypotheses as sequestrins in paper I inhibit aggregation by binding the monomeric conformation.

Amyloid cascade hypothesis

In 1992, Hardy and Higgins proposed the amyloid cascade hypothesis, in which Aβ aggregation into plaques leads to neurotoxicity and cognitive decline. The cleavage of Aβ₁₋₄₂ allows rapid assemble into fibril structures. Later studies have shown that the direct correlation between the disease and formation of plaques is vague. Fibril formation of Aβ₁₋₄₀ and Aβ₁₋₄₂ does however have different pathological effects, where the Aβ₁₋₄₂ fibrils are more neurotoxic. Signs of neuronal degeneration are observable before plaques start deposit. Earlier intermediate points of aggregation, as the smaller and soluble oligomers and protofibrils, show more toxic effects. This is the basis of the Aβ oligomer hypothesis (figure 1.3C).

Aβ oligomer hypothesis

Oligomers vary in size and include everything between a dimer and a dodecamer of Aβ, and it is believed to be a toxic specie of Aβ. Additional association of Aβ to the oligomer creates firstly a protofibril, and subsequently a larger fibril. It is debated if oligomers are in equilibrium with the monomeric Aβ concentrations or being an off-pathway intermediate before fibril formation. However, similarities in structural organisation between the different states are seen as they also are organised by an extended coil structure and a beta sheet.
Neurodegenerative disorders

Figure 1.6. A) The amyloid precursor protein (APP) with mutations common in AD. Cleavage site for $\alpha$, $\beta$, $\gamma$-secretase is marked. Mutations after position 691 affect the cleavage position of $\gamma$-secretase. In grey is the cell membrane spanning region of the protein and associated cholesterol in blue, with involved aa circled in black. Redrawn with modifications from Kant et al.[7] B) Partly structured soluble A$\beta_{1-40}$ with alpha helical region aa 13–23 (PDB:1BA6). C) A$\beta_{1-42}$ soluble structure with alpha helical content in aa 8–25 and 28–38, which is connected by a beta turn (PDB:1IYT). D) A$\beta_{1-42}$ pentamer with a parallel beta strand of residues 18–42 (PDB:2BEG). E) A$\beta$ fibril backbone arrangement, stacking with a steric zipper core interaction (PDB:2MPZ).
**Beta amyloid dysfunction (BAD) hypothesis**

In 2019, Hillen published the beta amyloid dysfunction (BAD) hypothesis which suggests the interaction of the Aβ with receptors by its monomeric, oligomeric, or fibrillar form induces neurotoxicity and is the reason for the disease development.\[16\] In the BAD hypothesis, FAD mutations are believed to change, the A\(_{\beta}^{1–42}/A\beta^{1–40}\) ratio (that is an important early biomarker for AD),\[21\] production levels, induce misfolding, and generate loss of function (LOF) of the A\(_{\beta}^{1–42}\). These changes and effects on homeostasis in the brain, are all reasons for disease development.\[16\] Since FAD mutations only account for 1–5% of the AD cases,\[3\] an alternative MOA is therefore suggested as the main reason behind the pathogenic behaviour of the peptide, where alterations as lower amyloid levels, LOF, and change in amyloid homeostasis are causative to sporadic cases.\[16\] Late-onset Alzheimer’s disease (LOAD) is a diverse mixture of factors of genetic and non-genetic factors. In LOAD, mutations in the ApoE alleles, presenilin-1 (PSEN1), or presenilin-2 (PSEN2) (encoding γ-secretase) are associated with degeneration, together with factors regulating A\(_{\beta}\) production and aggregation as well as decreased A\(_{\beta}\) clearance and degradation, increased inflammation, and resistance to γ-secretase activity.\[14\]

**Tau hypothesis**

Tau fibrils are commonly found in AD patients, and thus, an additional AD hypothesis is the tau hypothesis. Tau is a microtubule associated protein that is spliced into six different isoforms in the adult brain from the MAPT gene, with or without exons 2, 3, and 10. Insertion of the exon 10 adds a microtubule binding region with different number of repeats as three or four (3R or 4R tau) which is expressed mainly in the axons of neurons. In AD, the 3R and 4R tau are found hyperphosphorylated, which relate to the creation of neurofibrillary tangles (NFTs) in the neuronal cell body or neurofibrillary threads in the axons or dendrites.\[22\]–\[24\] Mutations in the microtubule binding region impair the function of tau and causes mis-localization and malfunction of microtubule regulation. Hyperphosphorylation stabilises the tau fibrils compared to normal tau, making them difficult to break down for the cell.\[25\] The creation and spreading of these fibrils are connected to AD progression and cognitive function impairments and may occur before A\(_{\beta}\) pathology is detectable. Tau fibrils are also found in other NDs (figure 1.4). Thus, tau accumulation is believed to be linked to neurodegeneration and has been observed to impact the cell transportation system, signalling system, and mitochondrial integrity.\[22\], \[26\], \[27\]
1.4.3. Current development of drugs for Alzheimer’s disease

The amyloid cascade hypothesis has for a long time dominated the field and thus, many of the therapeutics developed for AD have focused on decreasing the Aβ1–42 levels by e.g. β-secretase modifications as well as removal and inhibition of Aβ fibrils and plaque formation. But as new theories and novel understanding of the complexity of the disease have emerged, so have novel treatment and target options expanded. Today, therapeutics are targeting proteins and receptors such as, Aβ, tau, ApoE, lipoprotein receptors, and neurotransmitter receptors. Targets related to, neurogenesis, cell death, metabolism and bioenergetics, inflammation, oxidative stress, vasculature, growth factors and hormones, synaptic plasticity, the gut-brain axis, circadian rhythm, and epigenetic regulators are amongst others investigated.[13] Targeting several proteins or pathways simultaneously are emerging as well in combination treatments.

Among the 143 therapies currently in trials (as by January 2022), focus is on treatment of, cognitive enhancement (9.8%), neuropsychiatric symptoms (6.9%), symptom relieving (16.8%), and disease modifying treatment (83.2%). Treatment is done by using either small molecule drugs or biologics such as, antibodies, vaccines, and antisense oligonucleotides (ASOs). Among the disease modifying treatments (DMTs) 66.4% are small molecule drugs and 33.6% biologics. Of the DMTs 16.8% (28.6% in phase III) are solely focusing on amyloid pathology.[13] After the first DMT failure in 2002 by amyloid targeting immunotherapy,[28]–[30] several pursuits have followed with inadequate treatment responses. Today, only two DMT drugs (Aducanumab and Lecanemab) are currently approved (under an accelerated regulatory mechanism) by the US Food and Drug Administration (FDA) but no DMT is yet approved by the European Medicines Agency (EMA). Hopefully, more will follow in the future. A comprehensive review about the status of several novel therapies and approaches can be found in Cummings et al.[13] and a review about failed amyloid approaches in Panza et al.[28] Further focus herein is placed on antibody DMT approaches towards amyloid conformations currently in phase III trials and is summarised in table 1.2. The Aβ epitope and targeted confirmations differs between the therapeutic antibodies covered here, and from the sequestrins developed in paper I which targets the monomeric form of Aβ.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Abepitope</th>
<th>Affinity</th>
<th>Clinical trials</th>
<th>Status of clinical trials</th>
<th>AD population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aducanumab (Aduhelm)</td>
<td>Aβ fibrils, linear epitope aa 3–7</td>
<td>200 pM</td>
<td>pII: ENGAGE</td>
<td>Finalised, no significant change</td>
<td>MCI or Mild AD, Aβ PET+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: EMERGE</td>
<td>Finalised, significant cognitive decline reduction</td>
<td>MCI or Mild AD, Aβ PET+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIIB: EMBARK</td>
<td>Ongoing until 2023</td>
<td>Patients from previous trials</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIV: ENVISION</td>
<td>Ongoing until 2026</td>
<td>Early AD</td>
</tr>
<tr>
<td>Donanemab</td>
<td>Phosphodiesterase inhibitor</td>
<td>3–5 nM</td>
<td>pII: TRAILBLAZER-ALZ</td>
<td>Finalised, significant cognitive decline reduction</td>
<td>PET positive, cognitive decline 1–2 y, not severe</td>
</tr>
<tr>
<td></td>
<td>pyroglutamate form of Aβ31-42</td>
<td></td>
<td>pIII: TRAILBLAZER-ALZ2</td>
<td>Ongoing until 2023</td>
<td>High risk AD, PET-tau+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: TRAILBLAZER-ALZ3</td>
<td>Ongoing until 2027, placebo controlled</td>
<td>Mild AD, Aβ PET+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: TRAILBLAZER-ALZ4</td>
<td>Ongoing until 2023, comparing Aducanumab</td>
<td>Early symptomatic AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: TRAILBLAZER-ALZ5</td>
<td>Ongoing until 2025</td>
<td></td>
</tr>
<tr>
<td>Gantenerumab</td>
<td>Aβ Oligomers aa:</td>
<td>23 nM</td>
<td>pII/III: DIAN-TU-001</td>
<td>Prolonged until 2024, preventative</td>
<td>FAD: 30–50 y</td>
</tr>
<tr>
<td></td>
<td>1–11 or</td>
<td></td>
<td></td>
<td>Missed primary endpoint 2022 autumn</td>
<td>MCI</td>
</tr>
<tr>
<td></td>
<td>18–27</td>
<td>770 nM</td>
<td>pII: Gratitude 1/2</td>
<td>Ongoing until 2026</td>
<td>Preventative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pII: SKYLINE RO7126209</td>
<td>Ongoing until 2024, including TIR shuttle</td>
<td>Mild, prodromal to moderate AD</td>
</tr>
<tr>
<td>Lecanemab (Leqembi)</td>
<td>Aβ protofibril epitope aa 1–16</td>
<td>0.7 nM</td>
<td>pIII: CLARITY AD</td>
<td>Finalised, significant cognitive decline reduction</td>
<td>Early AD, Aβ PET+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: AHEAD DIAN-TU</td>
<td>Ongoing until 2027</td>
<td>Elevated Aβ, not impaired</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ongoing, together with anti-tau antibody</td>
<td>EOAD with FAD</td>
</tr>
<tr>
<td>Solanezumab</td>
<td>Aβ monomer aa 16–26</td>
<td>0.8–4 pM</td>
<td>pIII: EXPEDITION-1, -2</td>
<td>No significant reduction, only for subgroup mild AD</td>
<td>Mild to moderate AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIII: EXPEDITION-3</td>
<td>No significant effect</td>
<td>Mild AD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pII/III: DIAN-TU</td>
<td>Preventative, failed</td>
<td>FAD: 30–50 y</td>
</tr>
</tbody>
</table>
**Aducanumab**

The development of Aducanumab by Biogen and Eisai, started by isolating antibodies (box 2.2) from AD mutational carriers that had no symptoms of cognitive decline. One IgG1-antibody targeting the linear conformal epitope between amino acid 3–7 was found and has been the foundation for the developed therapeutic antibody that is today under accelerated approval by the FDA.\[^{31}\] The targeted amyloid epitope is available on all conformations of Aβ, the antibody however is specific for soluble oligomers and insoluble fibrils due to epitope density in combination with relatively fast association and dissociation rates. The high epitope density in oligomers and fibril targets results in avidity in the interactions, which increases the affinity from 9 μM to Aβ\(_{1-40}\) monomers to a 10,000-fold improvement in affinity for oligomers.\[^{31}, [32]\]

Today, the phase III trials ENGAGE and EMERGE that lead to the FDA accelerated approval for early-stage AD, are debated. Both trials were halted in an interim analysis in March 2019 where the primary endpoints for the studies were not met. However, in November 2019 after analysing the full data set of the trials, which included data until the day of the halted study (65.2% more data points), analysis showed a reduced cognitive decline in the high-dose (10 mg × ml\(^{-1}\)) arm for EMERGE with 22% in Clinical Dementia Rating Scale – Sum of Boxes (CDR–SB) score compared to placebo after 78 weeks of treatment. The addition of new data to the analysis showed a difference between the two studies. The ENGAGE study did not however yield any significant treatment effect, and an increased effect of treatment was seen with duration in EMERGE. This discrepancy led to the halting of the studies, as they were expected to have similar results and that treatment effect would be linear to duration of treatment.\[^{33}, [34]\]

In June 2021, Aducanumab was approved by FDA with an accelerated approval after the new data was reviewed. This led to the first new treatment option for AD since 2003, and Aducanumab became the very first approved DMT to AD.\[^{13}, [34]\] This decision has led to some critique against FDA, as guidelines demand two positive phase III trials for an approval which was neglected. The small differences in outcome between the ENGAGE and EMERGE studies are questioned if they contribute to a meaningful clinical effect, and if data are conclusive enough by only looking on the EMERGE trial.\[^{34}, [35]\] Additionally, the cost–benefit of treatment is also questioned for Aducanumab. The annual coast for treatment were initially set to $56,000 and was later reduced in 2021 to $28,200 after receiving critique.\[^{36}\] However, the estimated cost–effectiveness is only $2,500–$8,300 per year. Thus, the availability of treatment for the millions of patients with the diagnosis is
questioned. A post-approval confirmative phase IV trial, ENVISION, started in May 2022 and is expected to be finalised in late 2026 to verify the clinical efficacy of Aducanumab (table 1.2). EMA has rejected marketing approval of Aducanumab and the application for distribution within the European union by Biogen was withdrawn.

**Donanemab**

The antibody Donanemab is a humanized IgG1 monoclonal antibody specific for phosphodiesterase inhibitor pyroglutamate form of Aβ_{p3-42}. This antibody is targeting amyloid plaques with a nanomolar affinity and helps in clearing plaques from the brain by phagocytosis of microglia. Thus, it does not prevent deposition of new plaques or the growth of existing ones. The antibody has been shown to be strongly immunogenic and most patients developed anti-drug antibodies (ADAs) with treatment. However, at high treatment doses the patients’ Aβ levels declined under the amyloid-positivity threshold levels within six months. Additionally, cognitive decline was reduced with 38% for Alzheimer’s Disease Assessment Scale - Cognitive Subscale version 13 (ADAS-Cog13) in the phase II TRAILBLAZER-ALZ study.

In 2020, a phase III safety and efficacy study (TRAILBLAZER-ALZ2) started that will be ongoing for 76 weeks and results are expected in early 2023. In parallel, a phase III placebo-controlled study was initiated in 2021 (TRAILBLAZER-ALZ3) that will be ongoing until 2027. The TRAILBLAZER-ALZ4 trial comparing Aducanumab and Donanemab in different arms of the trial, started in November 2021 and is expected to complete in June 2023 (table 1.2).

**Gantenerumab**

Gantenerumab is a fully humanized IgG1 antibody binding to two different epitopes on Aβ. The epitope of the hydrophilic and soluble N-terminal at amino acids 1–11 has the strongest affinity of 23 nM, and a weaker interaction is seen in the central region of the peptide at amino acids 18–27. The antibody is mainly targeting oligomers and fibrils due to a decreased off rate with higher order of aggregates, leading to a 100-fold improved EC_{50} of aggregates. The rationale for treatment with Gantenerumab is that it will disassemble plaques, recruit microglia, and induce phagocytosis of the fibrils.

The antibody has been studied together with a β-secretase 1 (BACE1) inhibitor from Roche as a combination therapy but was later discontinued. The monoclonal antibody has been reported to lower the Aβ burden after...
36 months of monthly subcutaneous dosing.\cite{43} This study was in 2021 continued in a second open label study and is expected to be finalized in 2024. The phase III studies GRATITUDE 1 and 2 were investigating the reduction of cognitive decline in mild cognitive impairment (MCI) patients with AD. In November 2022, the studies however showed that the primary endpoint was not met. A reduction in cognitive decline of 8% and 6% in CDR–SB score, respectively, was observed which was not significant compared to placebo.\cite{44}

Interestingly, Washington University in St. Louis, USA, plans to start a preventative treatment for familial AD in persons as young as 18 years that have no previous Aβ burden. It builds on the phase II/III Dominantly Inherited Alzheimer Network Trials Unit (DIAN-TU-001) in which Gantenerumab showed improved biomarker values for treatment of FAD patients in the ages of 30–50 years. The trial is ongoing since 2012 and aims to prevent familial AD, by initiating treatment approximately 10 years before symptoms arise. In March 2022 a phase III trial was started, SKYLINE, with a four year long preventative treatment with the antibody dosed subcutaneously (table 1.2).\cite{38,45}

Due to the limited amount of antibody taken up into the brain, this antibody is currently investigated in combination with a brain shuttle technology to increase the brain availability (Trontinemab, RO7126209) (more info on the brain shuttle technology in chapter 2). Here, a Fragment Antigen binding (Fab)-domain targeting the transferrin receptor (TfR) is conjugated to the antibody, for increased uptake and it was the first antibody with a TfR-targeting domain to enter clinical trials. The study started in 2019 and results are expected to be reported in 2024.\cite{38}

**Lecanemab**

Lecanemab is a humanized monoclonal IgG1 antibody that is designed to target large soluble protofibrils of Aβ. It was developed to specifically bind the artic mutation (E22G) in Aβ that generates elevated levels of protofibrils of Aβ\textsubscript{1–40} and Aβ\textsubscript{1–42}, and reduced Aβ levels in plasma.\cite{46,47} It specifically binds the epitope formed by amino acids 1–16 in Aβ. The antibody has a 1,000-fold higher affinity to protofibrils (K\textsubscript{d} 0.7 nM) compared to monomers, and 10- to 15-fold higher affinity compared to fibrils.\cite{48–50} To increase the affinity and selectivity further, efforts to develop a hexavalent version of the antibody has been investigated.\cite{51} With six units that engages the target, the avidity effect for the Aβ increases, leading to decreased off rates between the hexamer and Aβ.\cite{51}
Neurodegenerative disorders

In phase II clinical trials in 2018, a reduced Aβ burden was seen together with a slowed cognitive decline of 26% CDR–SB at the highest dose of 10 mg × ml⁻¹ biweekly. In the fall of 2022, Eisai announced positive results from the phase III study CLARITY AD with reduced cognitive decline (27% CDR–SB) in early AD, with an effect starting at six months. A secondary phase III study AHEAD is running until 2027, and was recently accepted at accelerated approval by the FDA. The antibody is also investigated for preventative dosing in a DIAN-TU trial together with an anti-tau antibody for FAD-positive patients without any cognitive impairment.

**Solanezumab**

The antibody Solanezumab is an IgG1 humanized monoclonal antibody targeting the monomeric 16–26 amino acids in Aβ with picomolar affinity. It binds the Aβ monomer and prevents it from further aggregation, which is believed to shift the equilibrium between the different species of Aβ.

The antibody was investigated in a phase III (EXPEDITION-1 and -2) trial during 80 weeks of treatment by monthly infusion of 400 mg of the antibody. In the general trial, no cognitive difference was seen, but a reduced cognitive decline was seen for a subgroup with mild AD. The improvement occurred late in the trial and effect increased with time which is believed to be consistent with a disease-modifying effect. However, the benefit was low and as seen in the following trial EXPEDITION-3 for mild AD, no significant benefit was yielded. One hypothesis is that the presence of monomeric Aβ in blood might have mislead the interpretation of modest change in biomarker levels in CSF, in relation to the sink theory. In the sink theory the peripheral level of Aβ is linked to the level in the brain. Thus, the theory suggests that by targeting and decreasing peripheral Aβ level it could lead to a decreased level within the brain. However, concerns have been raised that Solanezumab does not recognize and do not bind to human Aβ in plasma from AD patients, which would limit its efficacy.

Gantenerumab, together with Eli Lilly’s Solanezumab, were evaluated by the DIAN-TU in a phase II/III trial aimed at preventing dementia in 210 people who are on the path to Alzheimer’s disease due to an inherited autosomal-dominant mutation in APP, PSEN1, or PSEN2 (table 1.2). Patients without symptoms did not show any cognitive decline during the period of preventative treatment. However, for patients that were pre-symptomatic, cognitive decline started before the target dosing level of the antibody was reached. Since the data was not conclusive, further adjustments are to be done in new trials such as, stratification on patient cohort by disease state, trial time...
starting 25 years before onset, and updated selection of biomarkers to follow the progression.[58]

1.4.4. Other treatments and targets for Alzheimer’s disease

With the questioning of the amyloid cascade hypothesis, an emerging number of other targets have been explored for development of therapies for AD. Other targets such as, tau, alternative epitopes on the Aβ, and combination treatments as e.g. Lecanemab with an anti-tau antibody are pursued. The use of antibodies or their derivatives are common for biologics, but other protein scaffolds are also emerging such as, catabodies,[59] nanobodies,[60] and alternative small scaffolds (chapter 4).

Another approach is using disaggregates and enzymes that can break aggregated forms of proteins. Engineered versions of heat-shock proteins (Hsp) 40, 70, 104, and 110 are investigated for several neurodegenerative disorders, and Hsp104 specifically for its Aβ1-42 disassemble functionality.[61]–[64] A human chaperone family that has been investigated for resolving aggregates of amyloid is the BRICHOS family, which has the benefit of natural BBB transportation (0.1–1%).[65], [66] The minor coat protein pIII found on M13-phage has a disaggregate function which helps in the infection of bacteria and has been engineered for therapeutic use in AD.[67] The active two subunits from the protein have been attached to a Fc-region and is used to reduce both Aβ levels and phosphorylated tau levels. It has been investigated in a first trial in humans where it was considered safe, but no effect was seen after six months dosing on patients with mild AD.[67], [68]

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A limitation for drugs that are targeting the central nervous system (CNS) is the small amount of the drug that becomes available in the brain upon intravenously or subcutaneous administration.[69] As for antibodies, other biologics, and most small molecule drugs, the passage into the brain is safeguarded by the BBB (chapter 2) and limits distribution into the CNS. Thus, it has been speculated that one of the limiting factors for effective AD treatment is the BBB passage and increasing brain uptake could yield higher therapeutic effect. This is investigated for Gantenerumab that only have a 0.07% BBB passage permeability, which is slightly lower than the generally considered BBB permeability for antibodies of 0.1–0.2%.[42],[70] It may further be interesting to investigate the effect of increased BBB passage for the mentioned antibodies and the effect on efficacy and dosing schemes, as explored for Gantenerumab/Trontinemab. However, depending on the target the low concentrations might still be sufficient.
Neurodegenerative disorders

The A\(\beta\) concentration of monomer in the CSF is believed to range between 1–10 nM and oligomers 1–100 fM.\textsuperscript{[71]–[73]} For achieving specificity for oligomers over monomers, around a 10,000-fold specificity is required, otherwise the drug will be trapped by a sink effect from the monomer conformation. This is displayed for Aducanumab, which only reaches nanomolar concentration in CSF, but still demonstrated efficacy due to its specificity for oligomers and fibril over monomers. If there is no specificity, higher doses are required for targeting the oligomer species due to the sink effect, and for targeting monomeric A\(\beta\), higher doses would also be necessary.\textsuperscript{[72]}

In contrast to the poor BBB permeability of antibodies the small molecule ALZ-801 has higher permeability and 40\% of the dose passes through the BBB in rodent studies, after reaching steady state dose. It is an oral drug, currently in phase III trials, which selectively interacts with the A\(\beta\)\textsubscript{1–42} monomers and prevents misfolding.\textsuperscript{[74]} Another approach is taken by A\(\beta\) vaccination, where several different epitopes are targeted. But so far, no treatments have shown positive effects in phase III trials and further efforts are ongoing.\textsuperscript{[29], [72], [75]}

Further on, the complexity of AD will probably require a combination of treatments for substantial effects on the disease progression. Starting treatment early, before the arise of symptoms is likely important for obtaining a disease-modifying effect. For this to be possible, good biomarkers and diagnostic methods are essential, but are not covered within this thesis. Classifying patient cohorts on genetic risk factors such as ApoE4,\textsuperscript{[76]–[78]} which might influence treatment efficacy, is also an intense research field. Until the underlying cause and pathways for both familial and sporadic AD are clearly understood, we will likely continue to see clinical failures. However, hopefully with the advancements in several fields surrounding AD, we can uncover new pathways and combinations for development of DMTs.

1.5. Parkinson’s disease

Today, the second most common ND is Parkinson’s disease (PD), which affects 1.2 million people world-wide, with increasing prevalence.\textsuperscript{[79]} PD is a multifactorial disease in which genetic and environmental factors are interplaying. There is evidence for protein aggregation causing failure of protein clearance pathways, mitochondrial damage, oxidative stress, excitotoxicity, neuroinflammation, as well as several genetic mutations correlated to the disease. Protein aggregates are often found intracellularly in neurons and consist of \(\alpha\)-synuclein, tau, and A\(\beta\). PD is multifactorial in its behaviour and is differentiated into disease subtypes such as dementia with Lewy bodies (DLB), and dementia with Parkinson’s disease (PDD) (figure 1.4).
The aggregation of Aβ (chapter 1.4) is linked to the severity of cognitive decline in PD, whereas α-synuclein deposits are connected to motor degeneration which is observed by the common symptoms of tremors and involuntary movements. The substantia nigra is the main affected area in the brain, where neurons are regulated by inhibitory dopamine (DA-neurons). Striatal DA-neurons found in this region are regulating the body balance. When the striatal DA-neurons are hyperactivated and cannot be turned off due to a loss of DA, they cause the involuntary movements, as seen in PD patients.[80]

1.5.1. Disease causations in Parkinson's disease

As in AD, PD is not completely correlated to one single disease mechanism. With the several PD subtypes, the disease-causing mechanism might differ, resulting in the range of symptoms observed in patients (figure 1.4). Since familial and sporadic cases have different non-genetic and genetic causes, they may also have different triggers for the disease.[80] Several hypotheses are thus presented for PD, with different relevance for different subtypes of the disease. In this thesis, focus is on α-synuclein as developed sequestrins in paper II target the monomeric form of α-synuclein and inhibits aggregation for several disease associated subtypes.

Functionality and genetic mutations in α-synuclein

Encoded by the SNCA gene, α-synuclein is aggregating in a similar fashion as Aβ. Aggregation is triggered from the hydrophobic NAC domain (amino acid 61–91) that change its alpha helical structure into a stacked beta sheet (figure 1.7). The monomeric form aggregates into oligomers and eventually fibrils by an unfolding of the original structure due to oxidative stress, aging, or environmental factors. Oligomers and protofibril aggregates of α-synuclein can cause a pore in the neuronal cell membrane, which leads to cell death.[80], [81] α-synuclein is enriched in the pre-synapse of neurons, where it mediates the release of neurotransmitters such as dopamine and interacts with the intracellular membrane (figure 1.8).[82], [83] Aggregated forms have been shown to create a synaptotoxicity as it reduces the synaptic plasticity of neurons. Lewy body pathology and neurodegeneration has in PD been linked to the synaptic spreading of extracellular α-synuclein.[84]

Familial mutations in the SNCA gene are correlated to early onset PD with dementia and fast progression of the disease.[86] There are, as in AD, SNCA mutations that have a subpopulation prevalence, A53T (Sweden, Greece, Italy, Australia, Korea), A30P (Germany), E46K (Spain), and H50Q (UK) (figure 1.7).[80] Overexpression of the SNCA gene yields a mitochondrial dysfunction
Figure 1.7. α-synuclein structures A) bound to the membrane in vesicles (PDB:1XQ8) within the cell where the region 1–91 aa form an alpha helical structure. The hydrophobic NAC region is determined by region 61–95. Marked positions for common disease related mutations are seen in the figure. B) Soluble structure of α-synuclein when not bound as predicted by AlphaFold2 CoLab[85] by Uniprot sequence P37840. The structure is a low probability structure as the unstructured version of α-synuclein is short lived due to the hydrophobic NAC region. C) Oligomeric structure (1×) of α-synuclein. The beta sheet formation triggers oligomerisation of α-synuclein (PDB:2N0A). Disease mutations are marked in light purple. D) Oligomer (10×) stacking by the beta sheet region leaving a unstructured C-terminal free (PDB:2N0A).
by aggregation located in the mitochondria. However, knock-out of the SNCA gene in turn, however, limits the membrane potential of the mitochondrial membrane and yields dysfunction and neuronal degeneration.[87] Mutations in correlated genes or at other positions than mentioned here may cause overexpression of the SNCA. Some SNCA mutations are correlated to specific behaviour in aggregation or disease as the aggressive A53T mutation which is linked to mitochondrial degeneration causing neuronal death,[80] or the A30P mutation which induces aggregation by exposure of the hydrophobic NAC domain in α-synuclein to the cytosol which leads to continuous propagation by detachment of the aggregate from the membrane.[88], [89] Further information about the lipid interactions can be found in reviews.[90], [91] The familial mutation E46K is correlated to specifically the DLB subtype (figure 1.7A).[92] Late onset PD is mainly correlated to familial mutations in the genes for, SNCA, DJ-1, parkin, and PINK1 where several mutations are known to correlate to disease development.[80]

**Figure 1.8.** Schematic illustration of the different structures and their physiological and pathological role. As by vesicle interactions, clustering, or aggregation by the free NAC domain interactions.
In addition to the NAC oligomerisation model, another mode of aggregation is hypothesised to form from the region of amino acids 36–57 in α-synuclein. This region is associated with several disease mutations in PD, and can form a β-hairpin structure that in turn trimerize into triangular shapes. The trimers, in turn, stacks into nonameres or related higher order oligomers (figure 1.9). By the stacking of trimers, the β-hairpin region becomes the centre of the aggregate rather than the NAC region (figure 1.7). The oligomers of α-synuclein are known to be polymorphic, and different oligomeric spices as suggested by the two models could co-exist and drive aggregation. The oligomers formed with the 36–55 amino acid region in the centre is inducing cellular toxicity by membrane disruption similar to the NAC oligomers. Since several mutations are correlated to this region and increases the aggregation propensity, other mutations have been found to decrease the aggregation propensity as e.g. L38M, Y39A, and S42A.

Sporadic cases of PD are correlated to the SNCA mutations A18T and A29S, and hyperphosphorylation of tau, as seen in AD. NFTs are often co-localised with α-synuclein deposits and contributes to the degradation of neurons. Additionally, environmental factors and impairments of the protein degradation system as the ubiquitin-proteasome system, autophagy lysosomal pathway, and a variety of Hsp are correlated to PD. The most commonly affected Hsp are 6, 40, 60, 70, 90, and 100 where several have correlations to AD and are investigated as potential targets for treatments.
**Prion hypothesis**

Spreading of pathological α-synuclein aggregates and the correlation to neuronal degradation is named the prion hypothesis.\[97\] The aggregated forms of α-synuclein spread in correlation to neuronal synapses from the diseased neuron, thus further infecting healthy neurons. Infection can start from neurons, or, as in the Braak hypothesis, from the gut. α-synuclein can, as viruses and bacteria, travel through the vagus nerve and enter the CNS from the gastro-intestinal tract and in a similar pathway enter from the olfactory bulb.\[97\] Since enteroendocrine cells can produce α-synuclein in the intestines and bacteria produces molecules or proteins that initiates aggregation (e.g. endotoxins or curli protein) it is believed in the Braak hypothesis that the soluble or aggregated protein can be taken up in the vagus nerve and be transported into the brain, where it spreads.\[98\] However, some objections have been made to this hypothesis as α-synuclein alone does not always cause PD.\[99\], \[100\]

**Dopamine and mitochondrial dysfunction hypothesis**

DA is needed as a neuronal signal inhibitor and as an antioxidant in the brain. By DA breakdown and by reaction with oxygen in the neuronal environment ROS are created. ROS are in turn affecting the mitochondria and induces cellular stress which contributes to the neuronal degradation. This leads to the mitochondrial dysfunction hypothesis in PD where both environmental and genetic factors are interplaying.\[101\]

**1.5.2. Current development of drugs for Parkinson's disease**

Since the complexity in PD is high, with few established causative factors, disease modifying treatments are lacking. Today, symptom relieving treatments for PD are used as treatment, which includes small molecule drugs which effects the dopamine levels and anti-acetyl cholinergic drugs to reduce the hyper activation of DA-neurons. In addition, some surgical symptomatic relief can be considered. For a potential disease modifying effect in PD, α-synuclein is targeted by active and passive immunotherapy.\[102\] Since there is a genetic link to PD, gene therapy is further investigated as a treatment option but is not covered within this thesis.\[80\], \[100\]

As by January 2022, 165 trials were ongoing targeting PD. Dopaminergic symptom relief is the dominating approach together with non-dopaminergic symptom relief. In the phase I trials DMTs are emerging such as targeting α-synuclein (9.8% of phase I trials) and glucagon-like peptide-1 receptor (GLP-1R) and 36% of the ongoing clinical trials are DMTs.\[99\] Another approach
Table 1.3. Selection of PD DMT antibodies currently in clinical trials. Further information about clinical trials is found at clinicaltrials.gov. Affinity for the specified α-synuclein (αSN) form or epitope is given for each antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>α-synuclein epitope</th>
<th>Affinity</th>
<th>Clinical trials</th>
<th>Status of clinical trials</th>
<th>PD population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prasinezumab</td>
<td>Aggregated αSN, aa 188–126 Monomer</td>
<td>48 pM, 20 nM</td>
<td>pII: PASADENA</td>
<td>Ongoing, open-label treatment until 2026. Failed early endpoints, but trend to improved motor functions</td>
<td>Mild PD, or newly diagnosed PD patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pIIb: PADOVA</td>
<td>Ongoing until 2023</td>
<td>Advanced symptoms PD</td>
</tr>
<tr>
<td>MEDI1341</td>
<td>Monomeric αSN and oligomers up to 200 kDa</td>
<td>74 pM</td>
<td>pI: NCT03272165</td>
<td>Completed in June 2022. No release of data</td>
<td>Healthy volunteers PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pII: NCT044449484</td>
<td>Completed in July 2022. No release of data</td>
<td>PD</td>
</tr>
<tr>
<td>Cinpanemab</td>
<td>αSN fibrils, aa 1–10 Monomer αSN</td>
<td>120 pM</td>
<td>pII: SPARK</td>
<td>Discontinued. Missed primary and secondary endpoints. Bound soluble protein in plasma with high injected dose</td>
<td>PD diagnosis &gt;3 y</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAN0805/ABBV-0805</td>
<td>αSN oligomers and protofibrils, aa 121–127</td>
<td>19 pM</td>
<td>pI: NCT04127695</td>
<td>Terminated due to strategic reasons</td>
<td>Newly diagnosed PD patients (&lt;5 y)</td>
</tr>
<tr>
<td>Lu AF82422</td>
<td>Monomer and fibrils (N- and C-terminal truncated forms)</td>
<td>20 nM</td>
<td>pI: NCT03611569</td>
<td>Completed in June 2021. No release of data</td>
<td>Healthy and PD patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pII: NCT05104476</td>
<td>Ongoing until 2023</td>
<td>MSA patients</td>
</tr>
</tbody>
</table>
Neurodegenerative disorders

is cell therapy, for instance implantation of DA-neurons or stem cells. The peptide phase III drug Exenatide targets the GLP-1R agnostically and is seen to have neuroprotective effects. It relieves the motor symptoms of the PD patients, and the clinical trial is expected to be finalised in 2024.

Treatments targeting the α-synuclein in PD are emerging, and DMTs are currently not beyond phase II trials. With the first results from DMT trails, some limitation in knowledge has been enlighten such as variations in endogenous α-synuclein levels between subtypes of PD, and the therapeutic window. Several active immunisation strategies to induce an antibody response in patients are currently in phase I and II for α-synuclein engagement. Passive immunisation antibodies targeting the C-terminal of α-synuclein is furthermore investigated. α-synuclein is normally predominantly located in the intraneuronal presynaptic terminal membranes and is important for the functionality of the presynaptic vesicle supply and release, creating part of the synaptic plasticity in neurons. However, passive immunisation strategies target extracellular α-synuclein, which is primarily spreading intercellularly by the prion hypothesis. Dosing the DMT without disturbing the natural function of α-synuclein needs to be considered for these therapeutics as the therapeutic window may be limited.

The humanised IgG1 antibody Prasinezumab is the first antibody that has been clinically investigated for targeting α-synuclein (table 1.3). It has demonstrated good tolerability and clearance of serum α-synuclein in phase I studies. This antibody has a higher affinity to oligomers of α-synuclein than monomers and targets the epitope 118–126. Extracellular aggregates are believed to be taken up and degraded by microglia via the Fc-γ interaction. However, the phase II study PASADENA was concluded not to yield any significant response for early-stage PD (table 1.3). There are currently several other antibodies targeting different domains and conformations of α-synuclein in early clinical trials such as, MEDI1341, Lu AF82422, Cinpanemab derived from healthy elderly individuals, and protofibril specific antibodies for e.g. the A30P mutation (BAN0805) (table 1.3). For the antibodies MEDI1341 and BAN0805, the effector functions of IgG are reduced by the use of an IgG4, as no immune response is desired. The MEDI1341 and Lu AF82422 are developed for high-affinity sequestration of monomeric α-synuclein.

The development of therapeutics targeting NDs are progressing, several pathways for neuronal degeneration and causations are scrutinised for both AD and PD. The current expanded knowledge and therapeutical development for these diseases will hopefully generate knowledge that can be applicable in
the future targeting of other NDs. Since many of the different NDs share proteinopathies, re-purposing drugs for other NDs and combination treatments are likely to increase in the future. The development of new DMTs for NDs face several challenges such as, selecting the appropriate MOA and combinations thereof, reliable diagnostics for early intervention, stratification of correct subgroups for treatment, and correct cognitive biomarkers for monitoring DMT effects. As has been shown for particularly antibodies in AD, the poor BBB penetration will likely continue to be an issue when targeting other NDs as well. BBB and approaches for increasing brain uptake will be further discussed in chapter 2.
CHAPTER 2
PASSAGE THROUGH THE BLOOD–BRAIN BARRIER

The sensitive brain environment is protected by the blood–brain barrier (BBB). It is a highly selective barrier of endothelial cells that controls the flux of compounds between blood and brain and only allows for passage of specific molecules. The BBB thereby protects the sensitive neuronal environment from potentially harmful substances or immunoreactions occurring in the blood. Targeting NDs with therapeutic proteins is thus a challenge since the passage over the BBB is restricted. The passage over the BBB is central in paper III–V, since they span the development of BBB shuttle and a model for transcytosis assay.

2.1. The blood–brain barrier

The brain is an extremely energy demanding organ. Approximately 20% of the body’s energy is consumed by the 100 billion neurons in the brain.\textsuperscript{112} To accommodate this energy demand, the blood vessels of the BBB are together over 600 km long and create a surface area of about 20 m\textsuperscript{2} to allow for sufficient transportation of essential compounds to support the brain.\textsuperscript{113} The endothelial cells that creates the blood vessels in the BBB have an overexpression of tight junctions that differentiates it from normal blood vessels. The neurovascular unit (NVU) that the BBB is part of, is created by the endothelial cells and the basal lamina, containing surrounding cell types such as, pericytes, astrocytes, and neurons (figure 2.1A).\textsuperscript{114} Pericytes help in regulating the blood flow, control the vascular development, and maintains the BBB.\textsuperscript{115} Astrocytes assist in forming the BBB by secreting factors that help in formation of tight junctions between the endothelial cells.\textsuperscript{116, 117} Neurons receive nutrients from the blood but also release hormones into the blood at certain locations in the brain. The basal lamina is produced by astrocytes and endothelial cells by secretion, and it surrounds the NVU cells on the abluminal side. The basal lamina separates the BBB cells from the brain parenchyma which consists of neurons and glia cells.\textsuperscript{116}

Within the brain parenchyma, the bulk flow or convection is very slow (0.2 µl × min\textsuperscript{-1}), and the main transportation is by diffusion. Thus, the distance from a capillary to a neuron needs to be small for nutrients to be able to reach
the cell. The brain parenchyma is filled with interstitial fluid (ISF) while the cerebrospinal fluid (CSF) fills the larger spaces within and around the CNS. Fluid is generated in the ISF from water diffusion by osmosis over the BBB. The water osmosis flow is 10-fold lower over the BBB than over a regular blood vessel due to the tight junctions formed by the endothelial cells. The ISF in turn partly drains further into the CSF and creates 10% of the CSF volume. About 400 ml of CSF is estimated to be produced daily in humans in the choroid plexuses. It has been observed that the CSF can flow into the brain parenchyma via specific routes, described as glymphatic circulation.

Since the total fluid volume in the brain is to be kept constant, the glymphatic system also drains excess volume out from the brain to the blood and lymph, a concept known as the Monro–Kellie dogma. A constant flow is important to maintain the neuronal cellular environment in an equilibrium between inflow of nutrients and outflow of cellular by-products that may create ROS.

As the blood volume within the brain varies with the cardiac cycle, the CSF flows into the brain parenchyma to make up for the loss in volume. This also helps in clearing out high-molecular weight compounds from the brain parenchyma. During sleep, the cardiac cycle is slowed down and a higher influx of CSF to brain parenchyma is seen, creating a faster clearance rate. With the constant influx back from ISF and constant creation of fluid in the CSF it will drain in several pathways as the arachnoid villi, perineural pathways, and spinal nerves leading into blood or lymph. Larger molecular weight compounds as albumin are removed by the lymph pathway and certain areas of the brain are more likely to drain into certain pathways. For example, ISF from the forebrain is less likely to mix with CSF and directly drains into the blood or lymph pathway. The complex interconnections between the fluids in the brain and fluid exchange between compartments and over different barriers are extensively reviewed elsewhere.

2.1.1. The challenge of delivering CNS targeted drugs

Very few therapeutic drugs become readily available in the CNS upon systemic administration. With several brain barriers therapeutic approaches to treat brain disorders face several challenges. Since neurons require nutrition and other essential compounds to function, these are transported across the BBB by several pathways. By utilising these natural transport pathways, a CNS drug could become available to the brain. Several means to develop molecules transported across the barriers have been investigated during the years and include strategies such as, receptor-mediated transportation, utilisation of influx transporters, inhibition of
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Efflux transporters, modulation of barrier integrity, or bypassing the barriers by opening them by e.g. osmosis, focused ultrasound, or intrathecal administration. For different influx and receptor-mediated transporters there are several different alternatives that have been investigated with varying success rate (figure 2.1B). Moreover, once the therapeutics have crossed the barrier into the brain the availability is likely limited due to slow diffusion in the brain parenchyma, glymphatic draining, and outflux by efflux transporters (figure 2.1B).

**Figure 2.1.** A) The neurovascular unit (NVU) creating the blood–brain barrier (BBB). The NVU consists of endothelial cells, pericytes, astrocyte feet, and neuron synapses. The side located in conjunction to the blood is the luminal side, and the side located to the brain is the abluminal side. B) Influx and efflux transporters at the BBB. Figure is created with inspiration and modified from Saunders et al.

In several CNS diseases (chapter 1) the integrity of the BBB is commonly damaged as an implication of the disease, causing a negative spiral. An example is in Multiple sclerosis (MS), where immune cells and cytokines can pass over the BBB when its structure is harmed and worsen the immune reactions in the brain – further damaging the BBB. In developed AD, the
integrity becomes weakened with both age and the disease progression.\cite{124,125} However, potential DMTs should likely be administered early in disease progression when the BBB is still mainly intact. Thereby, therapeutics targeting proteins within the CNS may benefit from an increased passage over the BBB.

### Box 2.1. The barriers of the brain

The brain has six different interfaces that function as barriers and protect the environment in the CNS. These include, the meningeal barrier, the blood–brain barrier, the blood–CSF, the circumventricular organs, the ependyma, and the embryonic CSF-barrier. The meningeal barrier is located at the meninges and separates the CSF from the dura by endothelial cells with tight junctions. The blood–brain barrier separates blood from the brain parenchyma by endothelial cells with tight junctions and the neurovascular unit. The blood–CSF barrier is found in the choroid plexus and is formed by epithelial cells with tight junctions at the apical CSF side, separating blood and CSF. The circumventricular organs barrier is created between the organ and CSF by specialized ependymal cells called tanyocytes that express tight junctions. It also separates the organ from the rest of the brain by an astro-glial cell barrier formed by tight junctions. Ependyma in adults separates ISF–CSF in brain areas. The embryonic CSF-barrier is a temporary barrier between the brain parenchyma and CSF, which is important for the developing brain. It develops in the adult brain into ependyma. As the barriers differ, they also require different mechanisms for transportation of nutrients etc across.\cite{122}

### 2.2. Pathways for crossing the blood–brain barrier

Nutrients and essential compounds are crossing the BBB using well-defined pathways such as, paracellular diffusion, adsorptive transcytosis, lipophilic diffusion, influx transporters, or by receptor-mediated transcytosis (RMT). Where the two latter ones are more commonly investigated for targeted BBB transportation for CNS pharmaceuticals of larger size. BBB influx transporters transport a range of nutrients into the brain such as, amino acids, glucose, and fatty acids (figure 2.1B). The RMT process includes transportation of for example, iron, insulin, and the hormone leptin.

#### 2.2.1. Adsorption and paracellular diffusion

Adsorption by the lipophilic pathway through the cell membrane is limited to small molecular weight compounds (<400 Da) with specific lipophilicity and is thus not relevant for biologics. However, another type of adsorption
transcytosis can occur for proteins as well. Endothelial cells have a glycocalyx layer on the luminal side that contributes to lowering the cell permeability. The glycocalyx consists of a negatively charged sugar-protein matrix expressing free sulphate groups and sialic acid. If a protein with a positive charge as e.g. albumin associates to the negatively charged glycocalyx, a clathrin and caveolin, or caveolin-mediated endocytosis process can be initiated. The glycocalyx is however a very unspecific process for BBB transcytosis as it is present on all endothelial cells. However, the glycocalyx on the BBB is displayed with an increased charge, adding to the increased low permeability compared to normal vessels. Adsorptive transcytosis is not preferably used as a specific transportation mode as the loading capacity is low, and as cargo usually becomes located in lysosomal compartments rather than being transcytosed. The glycocalyx effect on transportation of biologics is generally low but it has recently been investigated for targeted transcytosis by a variable lymphocyte receptor with nanomolar affinity. The effect of the glycocalyx on BBB permeability is however an important consideration for small molecular drugs.

Small hydrophilic molecules can pass though the BBB through the paracellular pathway. The solubility of these molecules and small size allows for travel between the endothelial cells and migrate through the tight and adherence junctions.

2.2.2. Passive and active diffusion

Passive or active transportation is achieved by specific receptors in the cell membrane on both the luminal and abluminal side. They transport the molecule into the cytoplasm and thereafter into the brain parenchyma. The difference between the active and passive pathway is the utilisation of energy to drive the transportation. In active transportation, ATP is required for initiation of the process as transportation is often against a concentration gradient. Passive diffusion often occurs with the concentration gradient or utilises co-transporters that goes in the direction of the concentration gradient. The expression of transporters for active and passive diffusion changes depending on capillary or venues, and area of the brain.

Sodium transport across the BBB is essential for maintaining the neuronal action potential. The ISF contains a higher concentration of the ion than plasma and thus needs transportation against the concentration gradient. By a combination of passive and active ion pumps this can be achieved, retaining homeostasis in the ISF. The transporters can also be specific influx transporters, as the transportation of glucose into brain from blood by the
influx transporter Glucose transporter 1 (GLUT-1). Others are specific efflux transporters, where transportation goes back into blood as by the P-glycoprotein transporter actively shuffling many substrates out of the cells and over the BBB from the brain (figure 2.1B). The P-glycoprotein is the main efflux transporter together with multidrug resistance transporters and generally shuttles small molecular weight drugs out of the brain parenchyma. An example is the PD drug Levodopa, which is actively transported into the brain by the L1 amino acid transporter. However, it is also actively transported out from the brain by sodium dependent transporters. Thereby, the available concentration in the brain parenchyma is dependent on the influx–efflux equilibrium and diffusion in the brain parenchyma away from the BBB.

2.2.3. Receptor-mediated transcytosis

Unlike active and passive diffusion, receptor-mediated transportation (RMT) endocytose the receptor with its cargo and transports it through or into the cell. Thus, if a RMT passes through the cell the cargo does not become available for the brain endothelial cell and is released into the brain parenchyma. Since RMT includes transportation of essential nutrients and signal molecules such as iron and insulin, the RMT in the endothelial cells allows for both cell internal uptake and BBB transcytosis. The RMT is however, unequally shifted in its distribution towards transcytosis at the BBB to allow for sufficient provision of nutrients to the brain. Since the RMT process is bi-directional the diffusion in the brain parenchyma aids in limiting the reverse transcytosis. The polarisation between luminal and abluminal side of the brain endothelial cells assists in favouring trafficking in a certain direction from either blood to brain or brain to blood for different receptors. For example, the neonatal Fc Receptor (FcRn) that binds to immunoglobulins is found at the BBB. It primarily transports antibodies from brain to the blood, as the frequency of FcRn RMT from blood to brain is low. A minor fraction of the RMT events results in lysosomal degradation of either both the content and receptor or only the receptor, following the endosomal maturation due to for example ubiquitin signalling to retain the protein homoeostasis (chapter 1). Thus, for each RMT process the receptor will eventually be transported back to the original membrane side, unless it is marked for degradation (figure 2.2).

Several receptors on the BBB are known to go through RMT. The receptors are generally not BBB-specific and are present in several other organs in the body. However, with the vast area of the BBB and with slightly increased receptor expression at the BBB capillary for some receptors, the possibility for directed
BBB transcytosis is increased. Currently, approximately 20 different receptors that undergo RMT are identified on BBB and include receptors such as, the TfR, the FeRn, the insulin receptor (IR), the leptin receptor (LEP-R), the apolipoprotein E receptor 2 (apoER2), and the insulin-like growth factor receptor (IGFR).[129]

The TfR has been extensively researched for its BBB transcytosis characteristics and high BBB expression compared to other receptors.[118] TfR is a homodimeric protein and receptor for the heavy chain ferritin complex (H-Ft) and transferrin (Tf) (figure 2.2). Both the H-Ft and Tf are iron carrying molecules for either cell storage or cell usage and minimise the toxic effects on cells from free iron. TfR has two stalks that anchor it to the cell membrane and allow for intracellular signalling. Upon binding of Tf to TfR, the receptor induces the formation of a clathrin-coated endosome and internalises. The newly formed early endosome has a slightly lower pH of around 6.5–7.0. From here several pathways are possible.[134], [135]

Firstly, the early endosome may be recycled to the cell surface, shuttled further to the Golgi for sorting, or further matured into a late endosome with a lower pH of 5.5–6.0. The lower pH for late endosomes will cause the iron carried by Tf (holo-Tf) to release. The released iron is pumped out from the endosome to the cell cytoplasm. This release of iron results in a conformational change in Tf into apo-Tf, which has low affinity for the receptor and thus releases from TfR. The pH in the endosome will increase again as it merges with the cellular membrane and apo-Tf is released into the blood to be loaded again with iron, while the receptor is fused to the membrane and available to bind a new holo-Tf (figure 2.2C).[134], [135]

Secondly, it has been shown that TfR can undergo a recycling pathway without endosomal maturation where it does not release iron into the cell. Thereby, holo-Tf is released back in blood or transcytoses over the BBB.[135]

Thirdly, a tunnelling pathway can be created for TfR that is quicker than the transcytosis process by endocytosis of the receptor (figure 2.2C). Thus, TfR has multiple pathways after binding Tf in the blood, but the control mechanisms directing the receptor to certain pathways are however still largely unknown. It has been shown that different Rab-proteins co-localises with different types of sorting events, as for example Rab17 co-localises with sorting tubule formation and transcytosis and Rab4a with vesicle formation and recycling.[136] About 5–10% of the RMT process for Tf will result in lysosomal degradation of the receptor and Tf, or only of the receptor.[135]
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A

Transferrin receptor

B

Transferrin receptor

C

Luminal side

Abluminal side

Recycling endosome pH 6.5–7.0

Recycled transferrin Fast pathway

Holo-transferrin bound to TIR

Early endosome pH 6.5–7.0

Recycling endosome pH 6.5–7.0

Degradation pathway 5–10%

Sorting tubule

Transcytosis

Late endosome pH 5.5–6.0

Transferrin iron release for cellular uptake

Apo-transferrin released

Lysosome pH 5.0–5.5
Several approaches for BBB shuttles have been reported utilising the TfR transcytosis pathway. Generally for these approaches, the endogenous function of Tf–TfR interaction should not be interfered with, as the iron delivery to the brain is essential. On the other hand, decreasing the iron uptake by blocking the Tf epitope has been investigated as a cancer treatment strategy, since TfR expression is often up-regulated on cancer cells.\[^{137}\]

Another endogenous TfR epitope is the heavy chain ferritin complex (H-Ft) binding site on the apical domain (figure 2.2A). TfR undergoes conformational changes upon binding to the H-Ft. This binding mechanism of H-Ft–TfR induces cellular uptake of iron, in which H-Ft functions as a cellular storage of iron in the cell.\[^{138}\] Due to the large size of ferritin and repetition of domains, it is believed that TfR commonly dimerises by binding the same H-Ft, which could correlate to the cellular uptake pathway together with the induced conformational change.\[^{139}\]

Due to the essential function of iron in cells, the TfR is expressed throughout the body as either the subtype TfR1 or TfR2 receptor, where the latter are predominantly expressed in hepatic cells.\[^{140}\] Thus, when targeting TfR1, potential off-target effects in other organs needs to be studied. Several affinity proteins have been described that are targeting the TfR without affecting the Tf uptake such as, antibodies, single-chain fragments, nanobodies, and small scaffold binders. This concept of hitchhiking with the natural ligand over the BBB without disturbing the endogenous function is known as a Trojan horse approach.
Figure 2.3. A) Crystal structure of the immunoglobulin (PDB:1IGT) and B) schematic view of an antibody with heavy (blue) and light chain (purple) regions, hinges, and receptor binding paratopes, and parts comprising the Fc, Fab, Fv, and CDR regions. The constant region includes the Fc and the first half of the Fab domain, and the variable the outer part of the Fab domain of both light and heavy chain. C) Different isotype classes of immunoglobulins with carbohydrates in grey circles.

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Box 2.2. Antibodies

Antibodies are produced by the human immune system as a response to infectious diseases and help in the adaptive immune response to clear out the infection. Antibodies can however be created to non-harmful compounds, leading to allergies, or to the body itself, known as autoimmune disorders such as MS and rheumatism arthritis. There are several classes of antibodies with different properties, effector functions, serum half-lives, valencies, and with different functions in the body. IgD is presented on B-cells and plays a role in induction of the antibody production. IgG has strong effector functions with long half-life and is the most abundant in sera. IgE is generated as a repose to allergies. IgM is main subtype in the first phase of the adaptive immune response and can form pentamers for an avidity effect to its target. IgA is produced and secreted into breast milk to help a baby’s immune response until they can produce antibodies themselves.[159]

Antibodies have a FcRn binding site allowing for recycling back to blood after endocytosis when filtrated out in kidneys. By the recycling, the half-life in serum is prolonged and less antibody needs to be continually produced by the body. Other important epitopes are the FcγR-family epitopes that is responsible for the antibody effector functions. This FcγR binding affinity is only present on the IgG subclass and enable activation of other innate immune cells such as e.g. dendritic cells, macrophages, and mast cells.[160]

The immunoglobulins (Ig) consist of two heavy and two light chains, which is divided into a constant and a variable region. The constant region changes depending on subclass and the variable region is yielding the target specificity. The two constant regions are connected in the hinge region by disulphide bridges. Different regions of the antibodies can be produced by protein engineering where a selection of the properties desired for the different regions determine the end format as a fragment crystallizable (Fc), a fragment antigen binding (Fab), a fragment variable domain (Fv), or a full Ig format. The complementarity-determining regions (CDRs) on each Fab consists of three loops with variability in amino acid composition and is determining the antigen specificity of the antibody and is usually also the randomised region in directed evolution (figure 2.3).[159]
2.3. *Trojan horse* delivery of CNS pharmaceuticals

By targeting the receptor and hitchhiking along the natural transcytosis pathway, drugs can be delivered over the BBB and to the CNS. Several of the mentioned RMT receptors have been investigated for this purpose with promising results such as, the IGFR, IR, and Tfr mainly using antibody derivatives. Small peptides fusions are however emerging, targeting for example the apoER2 and Tfr. Some candidates are currently evaluated in preclinical and clinical trials for BBB uptake such as the sdAb FC5 targeting a (2,3)-sialo glycoprotein for transcytosis, and the Tfr-specific shuttle from Roche/Genentech which was combined with Gantenerumab (Trontinemab) and the first Tfr shuttle to enter clinical trials. Other approaches include, the Denali/Takeda transport vehicle (TV) technology with a modified Fc-region for Tfr-mediated uptake that is currently in phase I trials targeting Hunters syndrome (MPS II) (DNL310), FTD (DNL593), and TREM2 in AD (DNL919). The IR-targeting antibody HIRMAB fused to the IDUA enzyme in Hurler syndrome (MPS I) is approved in Japan since 2021.

In BBB passage by RMT, different approaches to the scaffold design have been taken (figure 2.4). The RG6102 compound consists of a single-chain fragment antigen binding (scFab), of about 50 kDa, which is targeting the apical domain of the Tfr, fused to the Fc-region of the antibody Gantenerumab, creating a 200 kDa fusion protein. For the Tfr shuttles based on antibody derivatives, it has previously been shown that a monovalent Tfr-targeting improves the bioavailability in the brain. If a bivalent binder is utilized, the Tfr receptors might cluster, which leads to increased uptake, but a reduced recycling compared to the monovalent form. The added size of a bivalent compound bound to Tfr has been speculated to have issues undergoing tubular passage due to its size, which would lead to more frequent lysosomal degradation instead of transcytosis, which is observed for the homodimeric scFab shuttle from Roche. Clustering of the Tfr increases the concentration of intracellular adaptors that initiate the clathrin-mediated endocytosis and thus gives higher uptake. The TV from Denali is utilising the Fc-region (about 50 kDa) of an antibody, and combined with the knob-in-hole technology it only adds a small paratope for monovalent anti-Tfr binding to one of the constant chains (figure 2.4). In this manner, the FcRn and FcγR1 receptor binding epitopes are undisturbed and allows for fusion of the Fc to bivalent/bispecific Fabs or other proteins.
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Figure 2.4. PDB structures and schematic pictures for different types of BBB shuttles including the transport vehicle (TV) (PDB:1IGT), scFab (PDB:1IGT), scFv (PDB:2GJJ), nanobody (PDB:2X1O), and VNAR (PDB:4HGK). The size ranges between 11–50 kDa which is all smaller than a full antibody format of 150 kDa. In grey is the two CDR regions and hyper variable regions (grey) marked for the VNAR that differ from the human antibody with three CDR loops.

Even smaller derivatives of the antibody, only including the variable domain, or the variable domain from non-human antibodies have been utilized. The heavy variable domains from heavy-chain only lama antibodies (VHH), also known as nanobodies (12–15 kDa), have been generated for TfR-mediated transcytosis. Similarily, a shark variable domain from new antigen receptor (VNAR) TBX2 from Ossianix, have been reported, targeting the TfR for TfR transcytosis. Unlike the human variable chain with three complementarity-determining regions (CDRs), the shark VNAR have two CDRs and two hyper variable regions involved in the binding and is one of the smallest reported antibody derivatives module of only 11–12 kDa (figure 2.4). The smaller size of the TfR-targeting entity can lead to increased bioavailability in the CNS as they more easily diffuse in the brain parenchyma once past the BBB. This has been observed for the same antibody when changed into different antibody derivatives, which could be of importance when combining the TfR targeting entity with a therapeutic cargo protein.

In addition to antibody derivatives, smaller alternative scaffolds can be utilized for the same purpose and is further discussed in chapter 5, and techniques for selecting such molecules are covered in chapter 3. However, not
only size is important for the brain shuttle. If effector functions and reduced clearance by the FcRn receptor is desired, an Fc region might be suitable even if it results in a larger size. However, for some approaches effector functions are not desirable and a fusion of two small domains is more straightforward, and might allow for cost-efficient production in prokaryotic host and alternative administration routes such as subcutaneous injections.

Furthermore, the affinity and kinetics of binding is another important property to consider when developing targeted RMT, given that the shuttle must dissociate from the receptor after or upon crossing the BBB. This could be achieved by for example a fast dissociation or pH-dependent binding that results in release from the receptor during the endosome formation or maturation, similar to the pathway for Tf–TfR. Slow dissociation from the receptor has been shown to reduce the CNS bioavailability and leading to degradation in some cases. Furthermore, other studies have demonstrated that decreasing the affinity for TfR boosted brain uptake. It has also been shown in some studies, that high affinity and long exposure correlated with TfR degradation and cytotoxic effects. However, other studies have shown impressive brain uptake with high-affinity TfR-binders in the low nanomolar range, hence contradicting the previously suggested importance of low affinity. It has been discussed that the performance of TfR-directed brain shuttles is influenced by the interplay between affinity, kinetics, epitope, pH dependency, and valency, making the development of such binders even more challenging.

It has been reported, and especially for TfR, that the RMT processes decline with age along with reduced TfR expression, and that the RMT transport mechanism is partly replaced by adsorptive transcytosis by caveolin. However, since adsorptive transcytosis is less efficient, it will lead to a lower concentration of essential compounds in the brain. The lower expression levels and reduced RMT may therefore complicate the use of a Trojan horse approach in older patients.

In the development of TfR-targeting shuttles, cross reactivity between species is valuable for evaluations in preclinical models and translatability. However, the relatively low homology of TfR between animal model species and humans has made such studies complex (figure 2.5). The question of what the optimal affinity is, has been further complicated by the increased understandings of different expression profiles of TfR between species. For example, in a previous study on a TfR-shuttle in mice, a lower affinity was better since it reduced toxicity to reticulocytes. This was further correlated to an increased TfR expression on reticulocytes in mice and effector functions on
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the antibody. However, the “optimal affinity” was not translatable to primates or human in later studies where the TfR expression is lower on reticulocytes, and thus a higher affinity was tolerated. With the final use in humans the results in the model species for first evaluations and humans needs to be comparable, but also highlights the importance of good in vitro human models for early screening.

Figure 2.5. TfR1 receptor (PDB:1SUV) consisting of two disulphide-linked subunits, one subunit in grey and one in blue. The purple positions are non-identity regions (76% identity) between the human and mouse TfR1 gene based on uniprot data. Non-identity is seen in both the apical, helical, and protease-like domains of the TfR.

2.4. BBB models for studying TfR-mediated transcytosis

Utilising the correct models when assessing transcytosis capability for brain shuttle candidates is important before proceeding to clinical trials. Due to the limited translatability between in vitro models and in vivo models, many studies are directly screening for transcytosis in animal models. Thus, the use of mice models with or without human TfR expression has been frequently used. As briefly mentioned above, the intrinsic differences between mice and human expression of TfR may lead to development of a non-optimal human candidate. Thus, primate models as the cynomolgus monkey have started replacing mice models for transcytosis characterisation.

To mimic the BBB several in vitro models have been described with different levels of complexity. Most widely used is the transwell system with tissue culture inserts (TC-inserts) (figure 2.6A). These inserts can support growth of a brain endothelial cell line on a membrane. On the other side of the
membrane, another cell type can be introduced and co-cultivated. However, the thick, porous plastic membranes that is separating the two cell types has a relatively poor resemblance to the in vivo extracellular matrix and it has been seen that the cell lines may change their behaviour when cultivated on these inserts. Approaches to improve characteristics are investigated for the TC-inserts by for instance changing manufacturing techniques or the material.\[171\]–\[175\]

**Figure 2.6.** BBB models for in vitro usage. A) TC-insert with dual seeding of cells on apical (upper) and basal (bottom) side. B) Lab-on-a-chip model with separate flux of CSF and vessel compartments allowing influx, CNS interaction and efflux. Each compartment consists of different cell types, where influx and efflux have endothelial cells in the vessel compartment and astrocytes and pericytes in the influx-efflux side, the brain compartment consist of glia and neuronal cells and is dependent on diffusion within the compartment. Measurements can be taken from the BBB efflux and vessel 2 output. Redrawn with inspiration from Maoz et al.\[178\] C) Organoid model with an outer surface of endothelial cells and builds up inwards with the NVU cell types with a core of neurons. Diffusion/transportation is measured by sampling the core of the organoids after exposure.

It has been shown that applying a flow over the endothelial cells helps maintaining cell characteristics and promotes the induction of tight junctions. The flow is often integrated in lab-on-a-chip models, where several cell types can be combined on different sides of the membranes, and a BBB shuttle can be investigated for transcytosis during continuous flow over the endothelial cells (figure 2.6B).\[176\]–\[178\]

Cellular models mimicking the whole NVU are further emerging in the form of organoids (figure 2.6C). In organoids the model is a sphere, of which the outer surface consists of the endothelial cells followed by different layers of
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pericytes, astrocytes and in the centre neurons. The organoids can be used for studying transcytosis and effect of transported drugs on the NVU.\textsuperscript{[179]}

These \textit{in vitro} models are important, as an early selection and ranking between candidates for transcytosis capability is needed. This is further explored in a new simplistic model utilising recombinant silk protein as a nanofibrillar membrane for cultivation of brain endothelial cells in paper IV, and is further used for evaluation of candidates from a naïve selection towards TfR (paper V). In chapter 3, the directed evolution process is further explained for generating such binders.
In 1984, Eigen and Gardiner suggested a theoretical pathway for directed evolution of self-replicating RNA molecules.\textsuperscript{[180]} It was suggested that one could stepwise mimic evolution by starting out from a pool of different RNA molecules, separate the variants based on different criteria, amplify interesting candidates, and start over again with the new amplified pool of candidates to enrich for the desired variants.\textsuperscript{[180]} One challenge was to keep the linkage between the mutated genetic material and the expressed proteins. To circumvent this issue several display systems have been developed, as further covered in this chapter.

To generate biologicals for NDs and transfer over the BBB, selection systems for directed evolution are often utilised as a platform. There are several different scaffolds and proteins that can be used in the process of generating candidates for such purposes. During selection, one scaffold is often selected as a template structure. A library of different clones is created from this structure by randomising the amino acids in the scaffold, allowing for proteins with different characteristics to be generated. How the positions are selected is depending on the scaffold and library design, as covered in this chapter. The library members go through a selection process using a selection system of preference, to isolate library members binding a desired target with certain desired characteristics, which is covered in the second part of the chapter.

### 3.1. Library design for directed evolution

When randomising several positions at once, synergistic effects can be selected for (figure 3.1A).\textsuperscript{[181]} However, randomising all amino acids in a protein and exchanging it to every possible amino acid is not a feasible strategy when designing new libraries. If a protein of 50 amino acids is randomised by changing it to each amino acid (20) in each position the possible combinations will be $50^{20}$. This number is corresponding to almost $10^{34}$ possibilities, which is 5,000 times more than the unfathomable estimated number of bacteria and archaea on earth.\textsuperscript{[182]} Such libraries are simply not possible to handle and investigate. Instead, by knowing the structure and functionality of a protein, positions can be selected that are more likely to be important for target
engagement or functionality, which decreases the number of combinations in the library.\cite{181}

From the starting library the evolutionary pressure from selection may cause the protein to evolve into different directions. Ideally, the directed evolution would end in a global affinity maximum, but biases and unintended selection pressures might lead to a dysfunctional protein, or a local affinity maximum (figure 3.1).

**Figure 3.1.** Different library design strategies affect the size of the library. A) the randomisation area (blue) of the protein (circle), changes the library size depending on the number of positions randomised. Smaller libraries can be obtained by focusing randomisation to certain areas of the protein. The number of possible sequences define the sequence space. B) Different affinity maxima can be obtained, depending on the selection pathway. The local maxima might not be the global maxima in the sequence space. Several options for the starting (I) library are possible. Depending on the path evolution takes, local maxima may arise leading to the highest affinity for that combination or a dead pathway (II). However, it may not be the highest affinity possible for the scaffold if another pathway would have been utilised (III). C) In directed evolution, evolutionary pressure helps in selecting sequence motifs that are beneficial for the protein interaction. Diversification at a later stage may however not be possible since the evolutionary pressure has made certain motifs essential.
3.1.1. Rational design of protein libraries

Knowledge about the function and structure of the protein is crucial in rational library design. By selecting a few positions known for their importance in e.g. target engagement, and mutating these to only a few amino acids a smaller library, with hopefully a higher proportion of functional variants can be created. Importantly, amino acids that are not desired in a certain position can be excluded from the design, limiting the number of possible combinations.

This can be combined in a semi-rational combinatorial approach where a protein structure is randomised at positions that could engage in target binding, a so called site-directed saturation mutagenesis approach. Here, a defined mix of amino acids are introduced at specific positions of the predetermined region of the protein. This approach does not require predictions of the protein–protein interaction. Still, previously obtained knowledge about the scaffold is valuable in library design and have potential to increase the functionality. An example could be avoiding randomization of certain amino acid positions that are known to be important for structure stability, which would likely reduce the proportion of inactive clones in the library. This method is common for naïve library design and has been used in chapter 5 for a new protein scaffold.

In focused site-directed saturation mutagenesis, an even more limited selection of randomised positions is used, and the randomisation is often skewed in a certain direction. This method is suitable to use if a sequence is known that already bind the target, but characteristics need to be improved as in for example affinity maturation. Thus, the library design is skewed for the original sequence and designed to contain a certain number of mutations in the gene (figure 3.1).

3.1.2. Random mutagenesis

In addition to rational design, random mutagenesis can also be employed for generation of diversity in genetic libraries. Here, the positions and types of mutations are not controlled, and mutations can occur in any part of the protein and change into any amino acid or stop codon. One benefit of this approach is that positive serendipity effects on protein characteristics such as, stability, solubility, and affinity might happen. However, since certain amino acids, stop codons, or essential positions cannot be excluded, the number of inactive clones in the library are typically higher. A frequently used method for introducing random mutations is by using an error-prone polymerase during polymerase chain reaction amplification (epPCR) of the gene, which results in a genetic drift in the gene and can result in high
Table 3.1. commonly used display systems in directed evolution. The different systems are connecting the genotype (DNA) to the phenotype (protein). They differ somewhat in, possible maximal library sizes[^94] that can be generated, selection methods, target binding detection, and number of expressed proteins on their surface.

<table>
<thead>
<tr>
<th>Display system</th>
<th>Library size up to</th>
<th>Display of</th>
<th>Displayed on/by</th>
<th>Valency in display</th>
<th>Capture method</th>
<th>Tolerability to harsh conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage</td>
<td>$10^{11}$</td>
<td>Up to 100 kDa proteins[^99]</td>
<td>pIII, pVIII</td>
<td>1–5</td>
<td>Magnetic beads</td>
<td>High; Acid, UV, heat, reducing agents, proteases</td>
</tr>
<tr>
<td>Bacterial</td>
<td>$10^{11}$</td>
<td>Commonly up to 50 kDa, full IgG in periplasm[^99]</td>
<td>Outer cell membrane or periplasm</td>
<td>Multivalent (10^5–10^6 / cell)</td>
<td>MACS, FACS</td>
<td>Limited</td>
</tr>
<tr>
<td>Yeast</td>
<td>$10^6$</td>
<td>Up to 50 kDa</td>
<td>Cell membrane</td>
<td>Multivalent</td>
<td>MACS, FACS</td>
<td>Limited</td>
</tr>
<tr>
<td>mRNA</td>
<td>$10^{12}$–$10^{14}$**</td>
<td>Peptides / proteins 10–650 aa[^97, ^98]</td>
<td>Directly linked to mRNA</td>
<td>Monovalent</td>
<td>Magnetic beads, agarose, microfluidic chip[^99]</td>
<td>High</td>
</tr>
<tr>
<td>Ribosome</td>
<td>$10^{12}$–$10^{13}$</td>
<td>Up to 50 kDa</td>
<td>By Ribosome</td>
<td>Monovalent</td>
<td>Magnetic beads</td>
<td>High</td>
</tr>
<tr>
<td>Mammalian</td>
<td>$10^7$–$10^9$</td>
<td>Glycosylated proteins, larger protein structures. Full IgG[^94, ^200]</td>
<td>Cell membrane</td>
<td>Multivalent</td>
<td>MACS, FACS</td>
<td>Low</td>
</tr>
</tbody>
</table>

[^94]: Depending on transformation efficacy of bacterial strain
**[^98]: Decreasing library size with size of displayed protein[^99]
Directed evolution

affinity binders.\[185\] Although epPCR offers less control over the mutagenesis, the average number of mutations per gene can be adjusted by the number of PCR cycles and conditions in the reaction.

3.1.3. In silico design

With the increased knowledge of protein structures, protein folding, and sequence structure relationship, molecular modelling of proteins is increasing as part of the workflow in protein library design. With programs such as AlphaFold and Rosetta, protein structure and favourable mutations to proteins can be used to predict potential binders with desired properties.\[186\]-\[188\] The methods can be used as a tool in library design since it can aid the rational design.\[189\], \[190\] It can further be used as a first selection method by in silico screening and docking de novo structures to remove initial inactive clones from a library. Thus, the computational modelling is becoming a powerful tool in library design that can increase the overall library quality and hence the probability of finding high affinity clones during selection.\[191\]

3.2. Display systems

In 2018 the Nobel Prize was awarded to Frances Arnold and jointly to George P. Smith and Sir Gregory P. Winter. They had applied the theoretical work by Eigen and Gardiner into experimental procedures for directed evolution of enzymes, as well as phage display of peptides and antibodies.\[192\] After designing the library, the genetic sequence encoding the protein with the desired function is to be selected out from the library pool. Here, the importance lay in selecting the correct genotype together with phenotype presented in the selection system.

The directed evolution selection systems rely on the principle suggested by Eigen and Gardiner, where variants are separated and amplified, and used as a template in the next cycle.\[180\] After a couple of cycles, the genotypes remaining are identified and selected as single clones for in-depth characterisation of phenotypic characteristics (figure 3.2C).

The different systems commonly employed for selection usually physically connect the genotype and phenotype. Today, there are several reported methods, including phage display, mRNA display, ribosome display, bacterial display, yeast display, and mammalian display (table 3.1).\[193\] For naïve selections, a large library is desired since it allows for a higher probability of finding high-affinity binders. Following here is a broader introduction to phage display and Escherichia coli (bacterial) display as they are utilised in the work of this thesis.
Figure 3.2. A) Overview of phage particle and the different coat proteins present. B) Phage and phagemid vector overview and impact on valency by pIII display. C) An overview of one cycle of phage display. Bio-panning is usually repeated 3–6 rounds, depending on titers and selection strategy. Selection is starting from either the library or amplified phage stock from the previous cycle. Target antigen can be soluble or pre-captured during panning on e.g. magnetic beads for separation of non-binding clones. After extensive washing of the non-binding phage population, elution of phage is done by different strategies such as trypsin cleavage or by acid elution.
3.2.1. Phage display

Phage display is utilising filamentous phage as a carrier of the phenotype and genotype of the displayed protein. Phage is a virus that is only infecting bacteria and comes in many different subtypes. The most used types for phage display are f1, fd, and M13K derivative that are infecting *E. coli*. The M13K phage consists of five coat proteins, where pIII and pVIII are commonly utilised for display of recombinant proteins and peptides (figure 3.2A). However, pVIII is expressed in thousands of copies on the phage, which might lead to avidity effects during selection or issues with assembly of the phage particle.[202] Commonly, the pIII is utilised instead since it is only expressed in 3–5 copies per phage and tolerates large inserts up to 100 kDa.[195] Display on all five copies of pIII may reduce infectivity to bacterial cells, and thus a phagemid vector encoding the library is typically utilised instead of a full phage vector. The use of a phagemid allows for both double stranded replication (as a vector) as well as a single stranded replication for packaging into phage particles. Using the phagemid system, phage particles display a mixture of wild type pIII and pIII fused with the recombinant protein. Infection of bacterial cells using a phagemid vector requires the assistance of a helper phage to provide the necessary viral components to produce phage particles. The helper phage has a defective Ff ori to increase the incorporation of the phagemid gene. By utilising an amber stop codon in the phagemid encoding the displayed protein the distribution of displayed protein on the pIII can be directed to one of the five pIII to minimise avidity effects during selection and retain infectivity (figure 3.2B).[202]

Phage with the desired phenotype can be selected by incubation with immobilised target protein on a plate or by utilising magnetic beads with immobilised target protein. The target protein is thus often biotinylated to allow for capture on streptavidin coated magnetic beads (bio-panning). The phage library used in selection can be in the size up to $10^8$–$10^{11}$, where the maximal size depends on the transformation efficacy of the bacterial strain (table 3.1). The phage phenotype that binds the desired target can be captured with e.g. magnetic beads, while non-binders are washed away. The remaining binders are eluted from the beads by different strategies by for example trypsin cleavage of the displayed protein or by acid elution. Eluted phage are amplified in *E. coli* and new phage particles are used in the next cycle (figure 3.2C). By repeating this cycle usually 3–6 times (depending on phage titers, selection strategy, and sequencing method) and changing the stringency of the process, target-binding clones are enriched and will be present several times in the phage pool.[203] With an increased number of the clone, it can easier
Figure 3.3. A) *E. coli* display on the outer cell membrane using the AIDA transporter, together with elements for expression monitoring by ABD and a cleavage site for sortase (ST) and the displayed recombinant protein. B) The *E. coli* display system with display vector for multivalent expression in the bacterial host. C) A selection process utilising bacterial display with MACS and FACS approaches. A couple of cycles are repeated in MACS until the library size is small enough for FACS, and until a good target binding population is dominating in FACS. FACS gives a distinction between high and low expression and target binding clones.
compete during more stringent conditions for target binding among the other clones. By reducing target concentration this competition between clones can be increased, promoting selection of higher affinity clones. Utilisation of different strategies can help in directing the evolution of certain traits. For example by using off rate selection for high-affinity, pH-dependent selection or selection for other traits as solubility or thermal stability. Since phage are relatively stable, harsh conditions during the selection process can be used if desired, given that the target protein is tolerating the condition.

3.2.2.  

E. coli display

Bacterial display, of which E. coli display is a variant, is similar to phage display with display of the protein on its surface and encapsulation of the genetic material inside the cell. In contrast to phage, the larger size of cells allows for real-time monitoring of the selection process by fluorescence-activated cell-sorting (FACS). Moreover, utilisation of self-renewable cells obviates the need for infection of bacteria for amplification of enriched clones. The library size of bacterial displayed libraries are typically around $10^7$–$10^{11}$, and depends on the transformation efficacy of the bacterial strain (table 3.1). Unlike phage display in which monovalent display is preferred, the E. coli display format allows for $10^3$–$10^5$ copies of the surface-displayed recombinant protein. The number of displayed proteins per cell typically differs a bit depending on the producibility of the construct and on the type of surface transporter. In the E. coli display, an expression vector is transformed into the bacteria and encodes for the phenotype of the displayed protein. Within the vector the protein translation starts with a signal peptide for secretion of the protein to the cell surface. It could thereafter be fused to a normalisation tag as for example an albumin binding domain (ABD) from which the surface expression level can be monitored alongside target binding upstream of the surface transporter as the e.g. autotransporter Adhesin Involved in Diffuse Adherence (AIDA) (figure 3.3).

During selection from large libraries using an E. coli display system, a magnetic-activated cell sorting (MACS) step is commonly utilised, which is similar to bio-panning in phage display (figure 3.3C). MACS is then usually employed in a few rounds, as pre-enrichment, in order to reduce the library size to a diversity that can be screened in the flow cytometer. In order to screen for high affinity binders, FACS is usually employed. Here the target protein is fluorescently labelled, and the bacteria associated with a fluorescent signal can be sorted out with different stringent washes and gates set in the FACS. The fusion to a normalisation tag as the ABD allows for simultaneous...
monitoring of surface expression levels by co-incubation of fluorescently labelled human serum albumin (chapter 4) (figure 3.3C). After a couple of rounds, the genotype of the clones is identified and interesting clones are selected for in-depth characterisation.

Directed evolution is a powerful approach when searching for new therapeutic candidates and has been employed frequently. In chapter 4, selections of affibody molecules targeting Aβ and α-synuclein are presented, which were selected utilising phage display. In chapter 5, the use of directed evolution and different display systems is employed on a novel scaffold for NDs and to affibodies targeting the TfR.
CHAPTER 4
AFFIBODIES AS AFFINITY PROTEINS

As a complement to antibodies and antibody derivatives, small alternative scaffolds have been developed. The scaffolds are typically in the range of 5–20 kDa, comparable to or lower than the mass of a scFv. Several variants of alternative scaffolds have been reported such as, affibodies, DARPinS, bicyclic peptides, and anticalins.[215] The scaffolds are utilised in directed evolution to develop high affinity binders. The scaffolds have typically a predetermined binding region that can be engineered, and most have been reported to be compatible with several different selection systems. Inherent to their small size, the tissue penetration of alternative scaffolds is generally considered high.[216] With the smaller size, blood clearance is fast and no inherent activation of the immune system is generally yielded and must therefore be considered in development. Thus, they sometimes demand different solutions of protein engineering to overcome such limitations compared to antibodies.

4.1. Affibodies as an alternative scaffold

Affibody molecules are based on the engineered Z domain, which is originally derived from the IgG binding Protein A from Staphylococcus aureus.[217] The Z domain is around 6.5 kDa (58 amino acids) in size, with a structure composed of three alpha helices (figure 4.1A). The three helices have a hydrophobic core that stabilises the structure and has a fast refolding capability.[216] The Z domain is used as an alternative scaffold in directed evolution for development of affibody molecules.[217] Helix 1 and 2 creates a target binding surface that is engineered and 13–15 amino acids in this region are commonly randomised by site-directed saturation mutagenesis to create a library. In the design, certain amino acids are generally omitted such as, proline and glycine that break the alpha helical structure, and cysteines that create disulphide bridges and form multimers.

Affibody libraries have traditionally mostly been generated with degenerate NNN or NNK codons. Today, libraries are often synthesized with trinucleotide codons, which allows for relatively precise design of codon distribution in each position and minimises codon bias.[216], [218] The randomized positions in the first and second helix are within a DNA sequence of merely 78 base pairs,
which makes efficient synthesis in form of a single randomised oligonucleotide possible. Introduction of restriction sites or overlapping sequences for restriction-free cloning is thus easily included in the construction of the library oligonucleotide. The affibody libraries have successfully been used in several different display systems as E. coli display,[211],[213] Staphylococcal carnous display,[219]–[222] yeast display,[223] ribosome display,[224] and the frequently used phage display.[225]–[228] Affibody molecules against numerous targets have been reported in the literature, of which several display picomolar affinity or even lower.[216],[228]–[231] The developed affibodies have been evaluated for a variety of purposes as purification ligands,[225],[227],[228] molecular diagnosis tools in e.g. tumour imaging,[229],[233]–[236] or treatments of diseases.[220],[237] Depending on the purpose of the affibody, different traits are important such as, affinity, stability, retention in the body, clearance from blood, incorporation of unnatural amino acids etc. The affibody construct is possible to produce in multiple ways, depending on the desired functionality and design of the construct as by recombinant production in mammalian, yeast, and bacterial cell systems, or by chemical peptide synthesis.[238]–[240] Affibody molecules often tolerate relatively harsh chemicals and conditions due to their generally efficient refolding and high stability. Thus, affibody molecules are excellent for e.g. radio imaging that requires high temperatures for radiolabelling,[241] and purification where harsh alkali conditions are commonly used for regeneration of the affinity columns.[242]

Today, the affibody ABY-035 is in clinical phase II trials for several inflammation-related indications, including psoriasis. ABY-035 is a homodimeric affibody molecule that is specific for interleukin-17A (IL-17A), and the two domains are fused to an ABD to prolong half-life (chapter 4.1.1). The two identical affibodies bind the homodimeric target IL-17A with a high avidity, resulting in a subpicomolar apparent affinity, and inhibits the binding of IL-17A to the IL-17 receptor with high potency.[216] In the clinical trials, it has been shown to have a good safety profile and being well tolerated.[243] In fact, hundreds of patients have received ABY-035 biweekly up to three years without any side effects and with excellent efficacy. The small size allows subcutaneous administration while IL-17A-targeting monoclonal antibodies require intravenous administration.[243]

Another example is the human epidermal growth factor receptor 2 (HER2)-targeting affibody ABY-025 (Z\textsubscript{HER2}) that is in phase II/III trials for positron emission tomography (PET) imaging of HER2 positive breast cancer patients. The small size results in efficient tissue penetration and fast clearance in the
Affibodies as affinity proteins

body, which is suitable for PET diagnosis of cancer. Even small metastasis of HER2 positive cancers can be detected with the use of ABY-025.\textsuperscript{[233]–[235]} In preclinical studies, the $Z_{\text{HER2}}$ has also been investigated for therapeutic use as an ABD-fused radiotherapy agent, with prolonged half-life.\textsuperscript{[237], [244], [245]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_1.png}
\caption{A) The Z domain derived affibody molecule with three helices. Helices one and two contain 13–15 randomised positions (14 common positions are marked in beige) for generating naïve libraries. The hydrophobic interactions between the helices are keeping the structure in a defined format (PDB:2B89). B) The albumin binding domain (ABD) based on a Streptococcal protein G domain\textsuperscript{[246]} (PDB:1GJS).}
\end{figure}

4.1.1. Extending half-life in circulation

For most therapeutic purposes a long serum half-life of protein-based drugs has been considered desirable to limit the dosing frequency and thus increase patient compliance. Several approaches have been described to prolong half-life, such as FcRn recycling for antibodies, or by interaction to human serum albumin (HSA).\textsuperscript{[247]–[249]} HSA is with its 69 kDa above the size of the renal clearance and is recycled by FcRn, resulting in a half-life of about three weeks in the body.\textsuperscript{[250]} By associating to HSA the half-life of therapeutic proteins can be extended, and as an option the ABD was developed.\textsuperscript{[212], [246], [251], [252]} The ABD is a 5 kDa engineered protein based on the albumin binding region of Streptococcal Protein G (figure 4.1B).\textsuperscript{[212]} The engineered variant ABD035 has a femtomolar affinity for HSA\textsuperscript{[212]} and has as a fusion to affibodies shown to increase half-life up to days in circulation.\textsuperscript{[237], [253]}

As the affibody and the ABD are bacterial derived proteins, both scaffolds have undergone protein engineering\textsuperscript{[254]} to decrease the immunogenicity of the construct. The ABD has been further de-immunised (ABD094) to ensure tolerability upon administration and has so far proven safe upon
Additional administration.\cite{255} Additionally, it has been observed that fusion of the ABD construct to affibodies decreases the immunogenicity.\cite{256}

Several other reported strategies exist for prolonged half-life e.g. PEGylation, PASylation, and FcRn recycling.\cite{257} For example, an antibody or affibody can increase its half-life by, improving FcRn affinity on antibodies, introducing FcRn specific affibodies to a construct, or create fusion of affibodies to full antibodies (AffiMabs) to use the antibody FcRn mediated recycling. Further information can be found elsewhere.\cite{207, 258, 261}

4.2. Affibodies for neurodegenerative disorders

4.2.1. Targeting amyloid beta

Affibody molecules towards the A\beta_{1-40} peptide in AD have also been developed. The directed evolution yielded several binders, where the best binder (Z_{A\beta3}) had an affinity of around 320 nM, as measured in surface plasmon resonance (SPR).\cite{226} However, during the selection the evolutionary pressure had led to enrichment of cysteine-containing variants. In all confirmed binders, a cysteine was introduced at a specific position in the second helix which resulted in dimerisation of two affibody molecules. The dimer was further stabilised by the interaction to the target peptide and formed a stable complex.\cite{226} Additionally, it was seen that in the first part of the affibody sequence, several helix destabilising prolines and glycines had been enriched (figure 4.1). Nuclear magnetic resonance (NMR) spectroscopy revealed that this destabilisation resulted in changes in secondary structure upon target engagement and formation of a beta sheet in complex with A\beta (figure 4.2).\cite{262} The change in the helix structure led to exposure of the hydrophobic core of the affibody, allowing for interaction with the hydrophobic part of the A\beta peptide that formed a beta-hairpin between residues 17–23 and 30–36 (figure 4.2).\cite{263} This illustrates the strength of directed evolution, where non-envisioned functionality and combinations of mutations can be discovered after a selection. This new type of protein structure and mode-of-binding formed the basis for the development of a novel heterodimeric library in paper I (chapter 5).

The dimeric Z\_{A\beta3} was shown to bind the A\beta in a 1:1 stochiometric ratio and inhibited A\beta fibrillisation in ThT-monitored aggregation assays.\cite{226} The inhibition of A\beta aggregation was further confirmed in in vivo studies in transgenic (tg) AD flies.\cite{265} The expression of A\beta in the fly brain is toxic and generates phenotypes related to AD. Tg flies for A\beta_{1-40}, A\beta_{1-42} and the arctic mutant A\beta_{1-42,E22G}, respectively, were crossed with tg flies for the Z\_{A\beta3} monomer, Z\_{A\beta3} head-to-tail dimer, and a Z domain control. For tg flies
expressing Aβ_1-42 the life span was reduced. However, upon co-expression of the head-to-tail ZAβ_3 dimer, the life span was retained for Aβ_1-42 tg flies. For the artic mutation, almost a full retention of the life span was observed upon co-expression and the Aβ levels were reduced with 97%, correlating to the observed decline in neurotoxicity.[265]

Next, ZAβ_3 was designed into a head-to-tail dimer format with truncated N-terminal regions, stabilising scaffold mutations, and mutations in the target binding area. The new format was used in an affinity maturation effort using bacterial display and FACS for screening a semi-rational combinatorial library. Sorting the library generated several variants, and the best performing candidate was denoted ZSYM73.[221], [240], [266], [267] In the affinity maturation, the first generation dimeric ZAβ_3 that showed an affinity around 17 nM affinity as determined in isothermal titration calorimetry (ITC), was improved around 1,000-fold to a sub-nanomolar affinity.[230], [262], [267] This high affinity homodimer ZSYM73 was engineered for half-life extension (ZSYM73-ABD) and preventative effects on development of AD symptoms was studied in transgenic APP/PS1 mice. The APP/PS1 mice develop a cognitive decline with age, related to

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**Figure 4.2.** A) NMR structure of the homodimeric ZAβ_3 (PDB:2OTK) (blue and light purple) in complex with Aβ_1-40 (grey). The Aβ is sequestered and interacts with the hydrophobic core of the affibody. The cysteine bridge is marked as sticks between respective subunit in helix 2. B) Genetic sequence for Aβ with arrows for the residues involved in β-hairpin formation. C) Genetic sequence for ZAβ_3 with arrow for the beta sheet and alpha helixes and the truncated sequence in head-to-tail format.
Affibodies as affinity proteins

symptoms in AD pathology. In this study, the affibody was dosed three times a week for 13 weeks, starting at onset of pathology. The treatment prevented development of Aβ burden and cognitive decline, and no toxicity was observed. Immunocytochemistry further demonstrated lower amyloid burden in the brains of treated animals compared to controls.\textsuperscript{[230]} Based on this promising construct (Z\textsubscript{SYM73}) for inhibiting Aβ aggregation, other amyloidogenic beta sheet structures such as, tau, human islet amyloid polypeptide, and α-synuclein has been targeted for inhibitory mechanisms (figure 4.3).

Targeting the protofibrillar aggregation state of Aβ has also been pursued. The Aβ peptide sequestered in the Z\textsubscript{Aβ3} affibody resembles the Aβ peptide in amyloid plaques. In an engineering effort, a stable form of the Aβ protofibril was generated (Aβcc) by locking the antiparallel beta sheets in Aβ that does not form further aggregation products.\textsuperscript{[268]} By using this as a target antigen in a selection, protofibril specific affibodies were generated with low nanomolar affinities.\textsuperscript{[269], [270]}

![Figure 4.3. Homodimeric affibodies (grey) in complex with amyloidogenic peptides (light purple) A) Aβ (PDB:2OTK). B) α-synuclein (PDB:4BXL). C) Human islet amyloid polypeptide (PDB:5K5G). The length and structure of the beta sheet (originally helix 1) is differing between constructs (blue). Figure inspired by Ståhl et al.]\textsuperscript{[216]}

4.2.2. Targeting tau

Another hallmark of AD and other NDs is aggregated forms of the tau protein in the brains of patients. Tau has also been targeted with the homodimeric cysteine-containing affibody format for inhibition of aggregation. By protein engineering of dimeric Z\textsubscript{Aβ3} phage display selection from an epPCR library yielded a tau specific affibody-based binder called TP4. TP4 has eight amino acids substituted in the Z\textsubscript{Aβ3} scaffold and shows an affinity of around 260 nM, and no retained affinity for Aβ. Additionally, tau aggregation could be
inhibited in the presence of TP4.[271] The affinity is however probably too low for therapeutic or diagnostic use, and a higher affinity would be desirable.

4.2.3. Targeting α-synuclein

α-synuclein is correlated to the proteinopathology in PD (chapter 1.5). The region of α-synuclein that forms a beta sheet consists of amino acids 36–57 and can initiate amyloidogenic aggregation at physiological pH (figure 1.7).[93], [94] The epPCR library based on dimeric Z$_{Aβ3}^β$ was used in phage display selections against α-synuclein. The selection generated a binder called AS69, with four substitutions from Z$_{Aβ3}^β$ and interacting with the residues 37–54 that forms a β-hairpin in α-synuclein (figure 4.3B). Although the affinity was modest (240 nM), AS69 inhibited aggregation of α-synuclein at equimolar concentrations.[272] However, it was later demonstrated that the affinity of AS69 was too low for use in PET imaging of α-synuclein.[273]

AS69 has been evaluated for therapeutic use in preclinical models. Co-expression of AS69 restored locomotor function in a PD fruit fly model, with transgenic expression of α-synuclein_A53T.[274] The α-synuclein proteino-pathology can be induced by seeding of fibrils, and the therapeutic ability for modifying the disease is important even after onset of fibril formation. To model this pathology, preformed α-synuclein fibrils were injected in the brain of tg α-synuclein_A30P mice, which induced the degeneration of dopaminergic neurons in the striatum and substantia nigra. As in the fly model, a reduction of pathology and neurodegeneration could be observed when simultaneously injecting mice with AS69. The co-injection of AS69 with α-synuclein fibrils also reduced the astroglia response to the fibrils, correlating to the observed reduced neuronal damage. However, the microglia response was not affected by the AS69 injection.[275]

4.2.4. Multispecific construct design for ND targeted therapeutics

With the small size of affibodies, it is straightforward to genetically fuse several functionalities for recombinant production of multispecific proteins. As discussed in this thesis, therapeutic targeting of NDs generally requires a high affinity protein with high specificity. Drugs for NDs would further likely benefit from increased brain uptake and a suitable serum half-life. To enable these characteristics for a therapeutic affibody, it could be fused with another affibody targeting for example Tfr for BBB passage, and an ABD for extended half-life. Today, no such affibody construct has yet been developed and investigated, but steps towards this concept have been taken with this thesis.
CHAPTER 5
PRESENT INVESTIGATION

Recently, the first two DMTs for AD were approved by the FDA under an accelerated approval. Although the approvals were a breakthrough in the field, the demonstrated effects on disease progression are still modest, and for many other NDs no DMTs are available. Additionally, one general challenge with CNS-targeting drugs is the poor uptake to the brain,[69] leading to a need for high doses and risk for limited treatment efficacy.[74]

In this thesis, five studies are presented with focus on development of different types of affinity proteins intended for therapeutic use and increased BBB passage in the field of neurodegenerative diseases. In the first paper, the development of a new scaffold protein for selections towards intrinsically disordered aggregation-prone peptides is described. The scaffold was denoted sequestrin, and a sequestrin library was displayed on phage and evaluated by selection of binders towards the AD-related Aβ peptide. In the second paper, this new library was applied in phage display selections towards α-synuclein. In both studies, high-affinity binders with inhibitory effect on aggregation for respective peptide were identified. In the third paper, a previously developed binder for Aβ (ZSYM73)[212], [230] was genetically fused to an ABD[212] and to an antibody fragment for TfR (scFv8D3),[148] and the tri-specific protein was investigated for retained functionality of respective domains, as well as uptake to CSF.

In the fourth paper, a novel transwell method was adapted for screening of transcytosis over an in vitro model of the BBB. Herein, recombinant silk was utilised as a membrane in a transwell with characteristics similar to extracellular matrix (ECM).[276] The silk membrane supported growth of a confluent cell layer of an endothelial BBB cell line and the transwell assay was validated by showing transcytosis for the TfR-specific 8D3[148] antibody compared to a non-binding isotype control. Finally, in the fifth paper, phage display selections were performed using an affibody library towards TfR. The selection generated two cross-species TfR-reactive binders, and second-generation variants of the binders were created by rational design and characterised for potential compatibility as BBB shuttles.
5.1. Paper I – Construction and Validation of a New Naïve Sequestrin Library for Directed Evolution of Binders against Aggregation-Prone Peptides

Affibody molecules binds with a relatively flat binding surface to the target protein and high affinity binders can be generated to globular proteins. However, for smaller unstructured amyloidogenic peptides the surface area is reduced and with the flat binding area of affibodies, high affinity binders might be challenging to achieve. In previous efforts to develop an affibody molecule towards the Aβ peptide, a novel distinctly different affinity protein from affibodies was identified. The affinity protein comprised two domains in a homodimeric affibody linked together with a cysteine bridge and with substantially different secondary structure, as described in chapter 4. Further efforts have been made to reengineer this first-generation dimer construct to bind to other amyloidogenic peptides. The efforts demonstrated that it was indeed possible to engineer the specificity, but the affinities were modest. However, with the promise of targeting other amyloidogenic peptides and the specific sequestration of monomeric species, a novel heterodimeric library of sequestrins (Sq) was conceptualised in this paper. The sequestrin library was further investigated for utilisation in phage display selections towards amyloidogenic peptides. As a proof of concept, the library was initially used in selections for the monomeric Aβ peptide.

The design of the new sequestrin library was based on previous engineering efforts of the homodimeric Aβ-binding construct, implementing certain stabilising scaffold mutations, a N-terminal truncation, and a glycine-serine linker to form a head-to-tail fusion protein. The two subunits representing the scaffold were each designed to have eleven randomised positions (figure 5.1.1). The amino acid composition in the randomised positions was directed towards certain properties depending on the site of the mutation, with a predisposition of the amino acids found in the previously described binders. With independent randomisation of both subunits, the theoretical size of the library design yielded $1.27 \times 10^{22}$ possible combinations (figure 5.1.1) and the constructed phage library however, comprised $5 \times 10^9$ clones.

The library was designed with an average of seven mutations per sequestrin, and after five bio-panning rounds towards the Aβ peptide, the pool had four mutations on average. However, even if the sequence shift indicates a skewing of the sequence towards $Z_{SYM73}$, no clone that was identical to $Z_{SYM73}$ was seen after selection. In the initial library, a growth bias outcompeted the
amplification of phage with sequestrins in favour of monomeric constructs and bald phage. However, as selection proceeded the evolutionary pressure for target binding outcompeted the amplification of these non-desired sequences.

**Figure 5.1.1.** A) The affibody construct with randomised positions in beige (PDB:2B89). B) The Z$_{\beta3}$ scaffold in complex with A$\beta$ (grey) (PDB:2OTK). Each subunit is coloured in blue or purple. The cysteine bridge is shown as sticks between the subunits. C) The head-to-tail sequestrin scaffold with the 11 randomised positions in beige. D) The randomisation design of the sequestrin library with allowed amino acids in each position group. Reprinted with permission from Hjelm et al.; Int. J. Mol. Sci.; 2023.[279]

The clones found from the selections did all show a strong binding towards the A$\beta$ peptide as evaluated in SPR (figure 5.1.2). Slower off rates together with higher expression levels in production were observed for the clones originating from the last selection cycle. The two binders with highest affinity derived from this selection, Sq$_{A\beta22}$ and Sq$_{A\beta23}$, both had an affinity of 1–3 nM in SPR analysis, which is 100-fold higher compared to the original Z$_{A\beta3}$ construct with 320 nM in affinity in SPR analysis.[226] Additionally, the two sequestrins, selected from the new library, both showed a doubled improvement in producibility compared to the Z$_{SYM73}$ construct, further strengthening the design of the sequestrin library.

The first-generation binder towards A$\beta$ Z$_{A\beta3}$ showed a structural change upon target engagement.[262] This characteristic was also investigated for the novel high affinity sequestrins. A structural rearrangement upon target engagement was observed for the investigated clones, as expected (figure...
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5.1.3. The structural rearrangement was approximated from circular dichroism (CD) data. It was observed that upon complex formation between the peptide and sequestrin, a part of the random coil and for some sequestrins a part of the alpha helical structure was lost, compared to the peptide and sequestrin alone. The lost secondary structure was exchanged for mainly an increased beta sheet content of the complex, in accordance with previous observations for ZAβ.

Complex formation between the sequestrin and the peptide further appeared to increase the stability, as the thermal melting (T_m) point increased with more than 15 °C upon complex formation (figure 5.1.3C, D).

Figure 5.1.2. SPR sensorgrams for immobilised Aβ1-40 and sequestrin analytes at A–B) 25 °C in a 1:1.5 dilution series (342–30 nM) and C–D) 37 °C at 1:2 dilution series (300–75 nM) for 900 s. Panels shown for SqAβ22 and SqAβ23, respectively. The association rate is relatively slow due to the structural rearrangement that occurs upon target engagement. The off rate is in the 10⁻⁵ s⁻¹ range, which is around the limit for accurate measurement of the SPR instrument. At the higher temperature, the off rate is increased with a factor 10. Black curves are fitted curves for kinetic constants, coloured curves are referenced raw data. Reprinted with permission from Hjelm et al.; Int. J. Mol. Sci.; 2023.

Moreover, the selected sequestrins were found to efficiently inhibit amyloid aggregation of the Aβ peptide in a 1:1 molar ratio in ThT assays. Here, inhibition of both the Aβ1-40 and Aβ1-42 peptides was observed in presence of the SqAβ22 and SqAβ23. Together with the structural rearrangement, it indicates a
similar binding interaction between sequestrin and peptide, as demonstrated in previous NMR studies.[262]

Figure 5.1.3. Circular dichroism (CD) measurement of the Sq_{Aβ22} and Sq_{Aβ23} A–B) Structural rearrangement (green) of the sequestrin (blue) is observed upon exposure to the Aβ peptide (beige). Signal corresponding to the random coil (195–200 nm) is decreased, and beta sheet content is increased for the complex of Sq_{Aβ23} and Aβ (light purple). C–D) The thermal melting (T_m) point increases for the complex as the curves are displaying a T_m shift from 42 to 59 °C and 48 to 68 °C respectively for the two sequestrins in complex formation. E–F) Sequestrin structure in complex with Aβ (light purple) and full refolding (light blue) of the complex after thermal denaturation. Reprinted with permission from Hjelm et al.; Int. J. Mol. Sci.; 2023.[279]
With these successful results, it was also of interest to perform selections towards other amyloidogenic peptides in NDs such as, α-synuclein, tau, and TDP-43 involved in diseases such as, PD, AD, FTD, and ALS.

### 5.2. Paper II – Sequestrins inhibiting amyloidogenic aggregation-prone peptides

Today, the second most common NDs is PD.[79] PD is correlated to the molecular phenotype of α-synuclein aggregation, which has a similar initiation of fibrillisation as Aβ with formation of a beta sheet structure that initiates nucleation (chapter 1.5).[84], [93], [94] Thus, the sequestrin library constructed in paper I was utilised for panning against this amyloidogenic peptide, hoping to generate a sequestrin that could inhibit the aggregation process of the free monomer form. AS69 is a homodimeric affibody[272] that has previously been selected for α-synuclein with a relatively modest affinity of 240 nM (figure 4.3). It was however recently shown to have a promising effect in animal models by inhibiting α-synuclein aggregation, supporting the idea of specifically targeting monomeric α-synuclein for a potential disease modifying effect.[272], [275], [281]

After five cycles of selection by phage display, the sequestrin library revealed a different sequence profile, compared to the previously observed pattern for the initial library (figure 5.2.1). The sequence homology after four rounds converged at certain positions and the convergence was even more pronounced after the fifth round. Interestingly, although the sequestrin library was displayed in a heterodimeric format, binders with the same sequence in the first and second subunit were observed. This might allow for the same type of binding interaction to the target peptide by both subunits. However, the most enriched clusters displayed heterodimeric sequences that indicated a binding to the peptide in one specific orientation between the subunits and the beta sheet formed by α-synuclein. The sequestration of the hydrophobic α-synuclein beta sheet would allow for a stabilisation by the sequestrins hydrophobic core.[266] This was observed by an increased melting temperature of the complex between sequestrin and the α-synuclein peptide.[243]

Certain amino acids at specific positions have been shown to be important for binding between the homodimeric AS69 construct and α-synuclein.[266], [272] Some of these amino acids were enriched in the selected sequestrin pool, but only in the second subunit, suggesting a specific orientation between sequestrins and the target peptide. Since the α-synuclein peptide displays a heterologous beta sheet surface, the two subunits are in turn likely to have different interactions to the peptide. This is observed by the difference in
sequence distribution between the subunits (figure 5.2.1). For example, in AS69 the phenylalanine in the 20th randomised library position was previously identified as crucial for aromatic and nonpolar interactions with the α-synuclein.[272] However, among the sequestrins, this position was enriched for isoleucine in the first subunit, and for tryptophan in the second subunit, likely indicating a distinct interaction from each subunit to the target peptide (figure 5.2.1). The 16th randomised position in the second unit was also enriched for tryptophan, which was identified in AS69 as a crucial nonpolar leucine.[266]

![Figure 5.2.1](image)

**Figure 5.2.1.** Sequence comparison between the designed library and each subunit of the sequestrin after five cycles of selection rounds towards α-synuclein. Positions as the 6th, 16th, 20th, and 23rd have different characteristics within the selected phage pool and between subunits.

Another important nonpolar interaction is the 23rd randomised position within the library, which is in both units exchanged from valine to leucine. Valine and leucine are similar, but leucine has a longer carbon chain. In addition, the destabilised first helix (amino acid 5–8) is heavily mutated in the enriched sequestrin pool in comparison to AS69. This region is also different from the sequestrin pool selected towards Aβ (paper I) and skewed towards a more nonpolar amino acid distribution. The region has previously been observed to stabilise the monomeric and dimeric constructs by formation of salt bridges and hydrogen bonds, which help to create a conformational zipper structure.[266] However, with the nonpolar sequence shift in the beta sheet region, these interactions might be limited for several constructs in the enriched sequestrin pool. With the randomisation of this region and shift to nonpolar amino acids, the stability is however retained or improved for the investigated clones, exemplified by an average Tm of 44 °C for the selected sequestrins compared to the Tm of 38 °C for AS69.[274]

As in paper I, sequestrins from later selection cycles had a similar or improved expression profile, high affinity, and retained stability. From the enriched pool
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of sequestrins after selection ten clones were analysed. Five of these, $S_{\text{sqSN2}}$, $S_{\text{sqLSN3}}$, $S_{\text{sqLSN4}}$, $S_{\text{sqLSN6}}$, and $S_{\text{sqLSN11}}$ showed an affinity around 10–20 nM in SPR. In fact, the affinity from the sequestrins showed up to a 7-fold improvement in affinity at 25 °C compared to a head-to-tail dimer of AS69 (figure 5.2.2). Additionally, the sequestrins targeting α-synuclein changed their conformation upon target binding, which induced a stable complex with higher melting temperature.

![Figure 5.2.2. SPR sensograms with immobilised α-synuclein and sequestrins (Sq) as analytes at 37 °C. A) $S_{\text{sqLSN3}}$, B) $S_{\text{sqLSN4}}$, C) $S_{\text{sqLSN6}}$, D) $S_{\text{sqLSN11}}$, E) $S_{\text{sqLSN11}}$, F) AS69. Coloured curves are referenced raw data and black fitted curves. All analytes were run in duplicate at varying concentrations. Dotted lines are excluded replicates due to loss of functionality of the surface. A pump artifact was observed at 900 s.](image)

The five clones with best profiles from the SPR analysis were further evaluated for their capacity to inhibit aggregation of α-synuclein in a ThT aggregation assay and the head-to-tail dimer of AS69 was included for comparison. The sequestrins were tested towards the wild type α-synuclein protein and towards three of the most common PD mutations located in the $SNCA$ gene, A53T, E46K, and A30P[^80].[^88].[^89] These mutations display different aggregation characteristics where the more aggressive mutation A53T shows the fastest nucleation and fibrillisation. The sequestrins successfully inhibited the aggregation of all subtypes of α-synuclein at an equimolar ratio. The weaker fibrils formed by the A30P mutation were more potently inhibited by the sequestrins, also at lower molar ratios. At lower molar ratios of sequestrin (1:5, 1:10), differences in the ability to inhibit aggregation for the mutation variants
of α-synuclein were more pronounced. The head-to-tail dimer AS69, displayed a low efficacy for the aggressive A53T mutation, which was more efficiently inhibited by several of the new sequestrins (figure 5.2.3). Both the SqαSN2 and SqαSN4 were more potent in inhibiting the A53T mutation even at lower molar ratios, which is correlating to their higher affinities recorded at both 25 °C and 37 °C. Additionally, the SqαSN4 displayed an effect at lower molar ratios for several of the tested α-synuclein variants (figure 5.2.3).

5.3. Paper III – An Affibody Molecule Is Actively Transported into the Cerebrospinal Fluid via Binding to the Transferrin Receptor

In paper I and II, the new sequestrins demonstrated inhibition of aggregation-prone target antigens. However, for CNS-targeting therapeutics, BBB passage is also of importance and the uptake of sequestrins and affibodies to the brain is generally as inefficient as for antibodies.[230], [270] Thus, in paper III, the strategy of fusing an Aβ-binding affibody (ZSYM73)[230] to a BBB brain shuttle targeting TfR was investigated.

As brain shuttle, a scFv of 8D3 was used. 8D3 is a mouse TfR1-specific antibody, binding the apical domain of the receptor, and do not interfere with the endogenous mechanism of the receptor.[148] A bivalent scFv8D3 fused to an antibody has demonstrated up to 80-fold increase in brain uptake after 2 hours post injection in previous investigations.[161]

In addition to fusion with scFv8D3, the ZSYM73 construct was also fused to an ABD to prolong its serum half-life, since longer exposure has shown neuroprotective effect in transgenic mice in a previous study (chapter 4).[230] In this study, the tri-specific protein scFv8D3-ZSYM73-ABD, consisting of a single chain Fragment variable of the mTfR1 specific antibody 8D3 fused to the Aβ binder ZSYM73 and ABD was compared to ZSYM73-ABD (figure 5.3.1) for improved BBB uptake and compatibility of the shuttle with the sequestrin construct.

Herein, the addition of the TfR-specific entity for the scFv8D3-ZSYM73-ABD construct yielded a 9-fold increase in CSF-to-serum ratio (figure 5.3.1C). The tri-specific construct showed a CSF bioavailability of 1.43%, compared to 0.16% for ZSYM73-ABD after 24 hours in NMRI mice. The CSF-to-serum concentration ratio is not fully comparable to the bioavailability seen in CNS but can be considered as a correlation to the CNS uptake at the CSF maxima point which is reached at 24 hours.[282]
Figure 5.2.3. Aggregation inhibition assay for the sequestrin Sq₄SN₄ compared to AS69, towards α-synuclein proteins with different mutation phenotypes, A53T, E46K, and A30P. The inhibition assay was run at different molar excess of α-synuclein at a 1:1 (green), 1:5 (blue), and 1:10 (purple) ratio alongside a control of only α-synuclein (orange). Standard deviation as bars in respective colour in the graphs.
The fusion of ABD yielded in this case a half-life for $Z_{SYM73}$-ABD of 26 hours, which is similar to the half-life of murine serum albumin.[283] The half-life for the scFv8D3-$Z_{SYM73}$-ABD construct was however shorter, having a half-life of 7 hours. A shorter half-life has been observed for many other TfR-targeting antibodies and is likely due to TfR-expression in peripheral tissues such as liver and kidneys, hence acting as a sink.[162]

As this proof-of-concept study shows, affibodies and sequestrins are suitable as subunits for introducing different functions in multispecific drug candidates targeting NDs. A problem with 8D3 is that it only binds to mTfR, without cross-reactivity to hTfR. The lack of cross-reactivity limits this study to a conceptual preclinical study, where knowledge on this fusion protein is

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**Figure 5.3.1.** Schematic view of scFv8D3-$Z_{SYM73}$-ABD and $Z_{SYM73}$-ABD. A) Gene fusion schematic and B) schematic form of the tri-specific construct with the molecular weights for each unit and linker design between each unit. C) CSF bioavailability of the two different constructs in CSF to serum ratio after 3, 24, and 48 hours. CSF maximal concentration of scFv8D3-$Z_{SYM73}$-ABD was reached after 24 hours. Reprinted with permission from Meister et al.; *Int. J. Mol. Sci.*; 2020.[284]
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gained, helping in directing further studies. Paper V describes the first effort to generate affibodies targeting the TfR for BBB transportation, envisioned to eventually be conceptualised into one fusion protein of a ND-targeting sequestrin and an affibody for active BBB transportation.

5.4. Paper IV – *In vitro* Blood–Brain Barrier Model based on Recombinant Spider Silk Protein Nanomembranes for Evaluation of Transcytosis capability of biomolecules

During development of BBB shuttles, early and straightforward characterisation of transcytosis properties is valuable. Today, several methods are available for studying transcytosis, for example the transwell standards TC-inserts, as described in chapter 2.[171] However, especially the frequently used TC-inserts have a limitation in the low resemblance to tissue, which impacts the growth of some sensitive cell lines.[171] These cell lines might differentiate or lose characteristics upon cultivation and might thus not be representative for modelling the *in vivo* tissue behaviour. To circumvent this issue of low tissue resemblance other materials are investigated, such as e.g. spider silk. In this paper a novel adaptation of an *in vitro* BBB transwell model was developed and validated to create a BBB mimic, enabling studies of potential transcytosis of new brain shuttles.

By utilising recombinant spider silk functionalised with the RGD motif from fibronectin, a membrane surface similar to the ECM has previously been demonstrated to promote cell adhesion and cell proliferation.[276], [285]–[288] The functionalised silk self-assembles into nanofibrillar membranes with a thickness of 0.5–1.5 µm, which supports the cellular attachment and growth.[285] In a previous study, this recombinant spider silk has been utilised for modelling blood vessels, where endothelial cells were co-cultured with smooth muscle cells.[285] In the present study, the cell line was exchanged to a murine BBB endothelial cell line called bEnd.3, expressing the murine TfR. The silk membrane with bEnd.3 cells were characterised in terms of, the ability of bEnd.3 to form tight junctions, monolayer morphology, and transcytosis capability were investigated. The barrier formation capability for the bEnd.3 cell line is debated, as a relatively high permeability for small molecules has previously been reported. However, the use of bEnd.3 cells has previously been verified to be suitable for assessing transcytosis of proteins, for which it was utilised here.[289]

In this study, the antibody 8D3 was conjugated with the fluorophore fluorescein-5-isothiocyanate (FITC) and utilised together with a IgG2a-FITC or
IgG2a-AF647 negative isotype control antibody, with no reported binding to TfR or murine cells. The antibodies were tested for cellular binding, endocytosis, and finally evaluated in a transcytosis assay with the bEnd.3-silk membranes.

The 8D3 antibody was verified by flow cytometry to bind the bEnd.3 cells. To verify endocytosis of the antibody into the bEnd.3 cells, the pH dependent fluorescence of the FITC fluorophore was utilised to measure the pH environment of the internalised antibody. The observed signal corresponded to an environment of pH 6.11 ± 0.03, hence most likely in early and recycling endosomes.\[165\] Thus, the labelled antibody behaved as expected, by binding the endothelial cells followed by internalisation, which is required for transcytosis. The IgG2a isotype control did not bind the cells or was endocytosed and was thereby considered suitable as negative control.

To verify the functionality of the formed silk membrane with bEnd.3 cells, several parameters were investigated during cultivation such as the transendothelial electrical resistance (TEER), and tight junction formation by immunocytochemistry (ICC). For investigating the cell structure scanning electron microscopy (SEM) was utilised and sub-cellular structures were studied by transmission electron microscopy (TEM). After 8–10 days of cultivation, elongated bEnd.3 cells had formed a confluent monolayer on the silk. At this point, a higher TEER of 13 Ohm × cm², similar to reported values in literature, \[174\], \[177\], \[289\] was obtained for the confluent membranes (figure 5.4.1C). As previously verified in literature, the bEnd.3 cells can create a sufficient barrier up to the 6th passage. However, around passage 10, substantial decrease in TfR expression has been reported.\[174\] This was also observed for the silk membranes, and the TEER decreased for membranes with higher passage numbers (data not shown).

TEER increases as the barrier formation becomes tighter and at the higher TEER (reached after 8–10 days) tight junction formation measured by zonula occludens-1 (ZO-1) staining was observed to form at the cell interfaces (figure 5.4.1A). Endosomes and clathrin coated vesicles are important for the TfR endocytosis process.\[135\], \[290\] In ICC, co-localisation of the signal from the 8D3 antibody and clathrin was observed for cells on the silk membrane (*) (figure 5.4.1B). Additionally, 8D3 stained tubular structures in the cells was observed (**), which has been reported previously for TfR transcytosis.\[136\], \[291\]
Figure 5.4.1. Silk nanofibrillar membranes with bEnd.3 cells at high TEER (day 8) characterised by immunocytochemistry (ICC) A) for ZO-1 (red) expression, which is localised at the cell membrane. Cell nucleolus by DAPI in blue. Scale bar 100 µm. B) Endocytosis of the 8D3 antibody (red) is co-localising with clathrin (green) endosomes within the cell (*) and sorting tubule (**).
Cell nucleolus by DAPI in blue. Scale bar 50 µm. C) Scanning electron microscopy (SEM) of silk membranes with elongated bEnd.3 cells (grey) showing a confluent monolayer, with presence of cells with astrocyte-like morphology (purple) on-top of the membrane. Scale bar 100 µm. D) SEM imaging of a cross section of the membrane (blue) and apical side where cells and extracellular matrix (ECM) secreted by the cells are observed. Scale bar 1 µm. E) Transmission electron microscopy (TEM) showing an elongated cell with tight association to the silk membrane where thickness is depending on the position of the cell nucleus. Scale bar 20 µm. F) Different cellular structures and vesicles are observed within the cell. Tight junctions between the cells are expressed to a high degree and a tight association to the silk support is observed. Scale bar 2 µm.

SEM and TEM analysis of the bEnd.3 cells on silk membranes showed a monolayer cell formation and tight attachment to the membrane (figure 5.4.1). The silk membrane had the expected thickness of 1.1 µm and cells displayed both tight and adherent junctions. Clathrin-coated vesicles were observed again in TEM, strengthening the ICC results (figure 5.4.1F).

As the negative control antibody did not interact with the bEnd.3 cells, it was utilised as a control for leakage during the transcytosis studies of the 8D3 antibody.

First, a two-step method transcytosis assay was developed and evaluated. Here, the transcytosis of the 8D3-FITC was investigated together with addition of fluorescently labelled beads, used to monitor the integrity of the membrane. Both apical and basal media were analysed for the fluorescent signals after a 90-minute incubation of antibody. The membranes were thereafter equilibrated in fresh media for a defined period before the same membranes were analysed with the IgG2a-FITC isotype control antibody in the same manner (figure 5.4.2A). At high TEER, the formed membranes with bEnd.3 cells had a low apparent permeability ($p_{app}$) for the isotype control, correlating with values observed for non-transcytosed antibodies in other studies (figure 5.4.2A). The difference in $p_{app}$ between 8D3 and the isotype control increased with increasing TEER and barrier formation. At day 8, with high TEER, the $p_{app}$ values of 8D3 and IgG2a antibody differed significantly with a $p_{app}$ of $1.71 \times 10^{-5} \pm 0.07 \times 10^{-5}$ cm × s$^{-1}$ versus $6.76 \times 10^{-6} \pm 0.85 \times 10^{-6}$ cm × s$^{-1}$, with a p-value of 0.0002.

Secondly, a single-step transcytosis assay was developed for simultaneous measurement of barrier leakage and comparison to isotype control (figure 5.4.2B). Here, the control antibody was instead labelled with AF647, to allow for simultaneous readings in different fluorescence channels. Additionally, the fluorescent beads were omitted since ruptured membranes were easily identified by an increase in $p_{app}$ for the isotype control. Again, the two antibodies significantly differed in their measured $p_{app}$ with a p-value of 0.002 (figure 5.4.2B).
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Figure 5.4.2. The apparent permeability of the control antibody IgG2a-FITC (grey) and the actively transcytosed 8D3-FITC (blue) in a A) two-step method, at two assay time points during cultivation at high or low TEER. Dashed lines are the permeability over the bare silk membrane for the antibodies. At high TEER a significant (**p-value = 0.0002) amount 8D3 is transcytosed compared to IgG2a. A trend is seen already at low TEER (*p-value = 0.02). B) Single-step method: Simultaneous reading of the 8D3-FITC antibody and IgG2a-AF647, with a p-value of 0.002 (**).

The developed BBB model appear to be a promising option for early discovery phase screening of biomolecules for BBB transcytosis. A larger sample set of candidates could be screened even before the transcytosis assay, after further validation, by the endocytosis flow-cytometric screening. Thereby potentially reducing the number of proteins to be analysed in the transcytosis assay.

5.5. Paper V – Affibody molecules intended for receptor-mediated transcytosis via the transferrin receptor

Transport via the TfR is one of the most investigated and promising RMT pathways for increasing BBB uptake of drugs (chapter 2). Several approaches for targeting TfR have been described, for example using bispecific antibodies, VNARs, cysteine dense peptides, and engineered antibody Fc fragments. In addition to studies related to BBB shuttles, TfR has also been suggested
as a target for cancer therapy due to its overexpression on certain cancer cells and encouraging preclinical data using TfR-targeting monoclonal antibodies, scFvs, and probodies have been reported.\[^{137}, 293, 294\] For a cancer therapy approach, it has been discussed that targeting the Tf epitope on the receptor might be preferred, since it would potentially result in reduced proliferation from blocking of iron uptake. However, for BBB shuttles, the Tf–TfR interaction surface is generally avoided because it might impact systemic TfR functions, even if no studies have shown this so far in humans. Moreover, it would most likely substantially reduce the number of available receptors for transcytosis over the BBB.\[^{166}\]

In paper V, the objective was to develop a cross-reactive affibody molecules for murine and human TfR. From a phage displayed library, affibody molecules to the ectodomain of recombinant mouse and human TfR1 were selected in a cross-reactive manner. The identified sequences after selection showed relatively low sequence homology, with no obvious trends in the randomised positions. From the output of the different selection tracks, 19 candidates were selected for further evaluation. The candidates were first assessed for binding to native TfR presented on human and murine cells, to investigate cross-reactivity. Out of the 19 candidates, the majority bound to either human or murine TfR, and two clones were found to bind both human and murine cells. An additional screening criterion was that the clones should not share epitope with Tf, which was verified in flow cytometry for the two clones Z\textsubscript{TfR14} and Z\textsubscript{TfR18} (figure 5.5.1).

The optimal affinity when targeting TfR for increasing brain uptake has been intensely debated in the field\[^{143}, 161, 167\] and one argument is that transcytosis is more efficient with lower affinity and in particular with a faster off rate. It is also partly connected to the valency of the affinity protein, as bivalent binding has been shown in some studies to interfere with transcytosis and lead to lysosomal degradation.\[^{136}, 143, 161\] However, examples of monovalent alternative scaffolds and antibody derivatives with relatively high affinity for TfR (low nM range) have more recently been shown to efficiently transcytose over the BBB.\[^{146}, 162, 295\] The affinity for the developed affibodies Z\textsubscript{TfR14} and Z\textsubscript{TfR18} were found to be 90 and 170 nM to murine TfR, respectively, and to human TfR 150 and 710 nM in SPR analysis. However, both variants displayed both a quick on rate and off rate and quite low thermal stability and tendencies to aggregate. Therefore, further rational engineering efforts were performed to potentially identify higher affinity binders with primarily faster on rate.
Figure 5.5.1. The effect of transferrin-Alexa Flour 488 (Tf-488) in molar excess on affibody signal for A) ZTfR14-ABD or B) ZTfR18-ABD. Where the affibody signal without Tf (purple) was maintained at different molar excess of Tf-488 (blue). C–D) In the same manner was the Tf-488 signal shift increased with concentration. The two highest concentrations of 60 and 85 µg × ml\(^{-1}\) of Tf-488 are in the case of ZTfR18-ABD at a lower cell count in both fluorescent channels. The signals do however, have a mean of fluoresce intensity (MFI) that aligns with saturation levels observed in the same range as ZTfR14-ABD measurements. Tf-488 binding was not affected by the presence of affibody.

For this effort, a single amino acid mutagenesis scan was employed where each randomised position in both ZTfR14 or ZTfR18 scaffold was mutated to the amino acid of the original IgG-binding affibody\(^{[217]}\) or to a histidine to promote pH-dependent binding.\(^{[206]}\) Additionally, the mutagenesis would possibly resolve some of the previously observed issues with poor solubility, limited production yields, and low T\(_m\). These small libraries were displayed on E. coli (chapter 3) and screened for improved binding and expression levels on the bacteria using a flow cytometric set-up (figure 5.5.2). For each candidate, a panel of mutations with a somewhat higher affinity for TfR were identified. Several mutations were observed to impact both the apparent affinity and the expression level in the E. coli display format. Two mutations were further analysed in soluble format (figure 5.5.2). Furthermore, the two mutations for each candidate were also combined in double-mutants to investigate potential synergistic effects. Additionally, as a negative control the Z\(_{\text{HER2}}\) affibody was included as a negative control.
Figure 5.5.2. Flow cytometric screening of *E. coli* displayed clones in comparison to the original variant for the improved variants ZTFR#14_A9H and ZTFR#14_A9H L27H or ZTfR#18_I11N and ZTfR#18_M14H. The x-axis represent an estimation of the expression levels by HSA association to expressed ABD on the cell, and y-axis target binding to human TfR. The ZTfR#18_M14H mutation seems to impact the expression level of the *E. coli* display.

In the soluble format, the mutated variants were verified to have a slightly improved $T_m$ in comparison to the original binders. In similarity to paper IV, the endocytosis assay investigation using FITC labelled binders was employed again. The mutated clones were generally correlated to different endosome compartments rather than to lysosomes or the cell surface and grouped with the original clone (table 5.5.1).

To evaluate these candidates in a transcytosis setting, the single-step transwell assay with recombinant spider silk from paper IV was utilised. To control for potential biases from molecular weight, the ZHER2 affibody was included for comparison. In the assay, it could be observed that all but the ZTfR#14_A9H and ZTfR#18 Affibody were transcytosed over the bEnd.3 barrier, of which three were of higher statistical significance. In the endocytosis assay, ZTfR#14_A9H displayed a higher pH environment and is likely still bound to the cell surface to a large degree (figure 5.5.3). The ZTfR#18 construct showed a lower pH environment in the endocytosis assay and may potentially partly get trapped within the cell.
but is still transcytosed. Although the in vitro assay indicated that several of the affibody variants underwent transcytosis to the basal side, this needs to be verified in vivo. Correlation between the endocytosis data and transcytosis capability further needs confirmation in vivo before it can be used as a primary screening method.

Table 5.5.1. Estimated pH from experimental samples of $Z_{\text{TR}}$-FITC. The different affibody type $Z_{\text{TR}14}$ or $Z_{\text{TR}18}$ groups by the original clone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calculated pH environment by standard curve</th>
<th>Vesicle classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>(HE)$_3$-ZHER2-FITC</td>
<td>5.75 ± 0.02</td>
<td>Late/lysosomal</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR14_A9H-FITC</td>
<td>7.15 ± 0.31</td>
<td>Vesicle</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR14_L27H-FITC</td>
<td>6.94 ± 0.02</td>
<td>Early</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR14_A9H_L27H-FITC</td>
<td>6.18 ± 0.04</td>
<td>Recycling</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR18_I11N-FITC</td>
<td>5.13 ± 0.02</td>
<td>Lysosome</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR18_M14H-FITC</td>
<td>6.56 ± 0.02</td>
<td>Early</td>
</tr>
<tr>
<td>(HE)$_3$-ZTR18_I11N_M14H-FITC</td>
<td>5.16 ± 0.01</td>
<td>Lysosome</td>
</tr>
</tbody>
</table>

Figure 5.5.3. Apparent permeability ($p_{\text{app}}$) for transcytosis over a bEnd.3 cell barrier formed on recombinant silk. Simultaneous reading of the affibody and internal control (IgG2a). All the $Z_{\text{TR}}$ constructs but the A9H variant is transcytosed with significance of "**p-value<0.05", "***p-value<0.01", and "****p-value<0.005". The $p_{\text{app}}$ of the internal control might differ slightly due differences in barrier formation for each membrane used in the analysis.

Screening of individual mutants on *E. coli* was straightforward, resulting in data on both affinity and expression levels without the need for purification of the around 50 different candidates. By only changing one single amino acid, a clear difference in properties could be seen for refolding, thermal stability, solubility, and binding capacity to the receptor.

However, the affibody candidates still need some optimisation in producibility, and further maturation is likely needed. For such endeavours, early screening methods for transcytosis that are utilised here would be beneficial. Again, the transcytosis capability in the transwell assay suggests an
active uptake, but this needs to be verified in in vivo models together with biodistributions studies. The binders presented here have retained cross-reactivity for murine TfR, allowing for preclinical investigations in mouse models. In the future, evaluation of cross-reactivity to other species, such as cynomolgus monkey that has a BBB with high similarity to human BBB, is of further interest.

5.6. Conclusions and further objectives

This thesis has been addressing several challenges related to development of DMTs for NDs. Despite huge efforts worldwide, many of the NDs are still poorly understood and the underlying disease pathways may be several. Finding new treatments has thus been cumbersome and the best drug targets are still not fully concluded. When developing CNS therapeutics, another challenge is to deliver high enough concentration of drugs into the brain. RMT shuttles utilising the TfR has been investigated by several research groups, and the first BBB shuttle together with a DMT antibody for AD is currently in clinical trials.[152], [153]

In the first and second paper, a novel heterodimeric affibody scaffold, denoted sequestrin, was developed for targeting monomeric aggregation-prone peptides. The amyloidogenic peptides $\alpha$-synuclein and $\beta$-hairpins that initiate aggregation. Sequestrins from phage display selections were shown to be able to sequester the monomeric form and thereby inhibit further fibrillisation. How these candidates will function in an in vivo setting will be interesting to evaluate, and further studies would need to be undertaken to understand the important interactions between the sequestrins and respective peptide. In the third paper, the addition of a BBB shuttle was shown to increase the concentration of a sequestrin in the brain. What is important to consider is the disease pathway, as the therapeutic drug may or may not need to be shuttled over the BBB for a therapeutic effect. When targeting the monomeric form of $\alpha$-synuclein, there is also monomers present in blood, creating a potential sink. Thus, delivering additional amount to the brain by a BBB shuttle strategy may not yield the desired improved efficacy. Even with a limited brain uptake, the $Z_{SYM73}$-ABD has shown neuroprotective effects.[230] An experimental therapy study, comparing $Z_{SYM73}$-ABD with and without a BBB shuttle is therefore of interest.

In the case of Aducanumab, the low BBB permeability has still yielded (although debated) clinical response. With a high dose, sufficient amount of antibody can pass over the BBB to yield therapeutic effect for a low abundant target as $\alpha$-synuclein fibrils.[33] However, when targeting more abundant proteins, or if
the high dose is not tolerable, *Trojan horse* approaches may be beneficial.\cite{72} It should also be noted that even if the drug is tolerated in clinical trials, with the slow disease progression of many NDs and the limited efficacy of today’s drugs, patients will likely need treatment for several years or even decades, which might be problematic. With the complexity of NDs, limited species translatability, and the multiple possible treatment pathways, development of novel therapeutics is challenging. Thus, in development, different types of drugs and different target conformations needs to be considered when evaluating development of new treatments in the future.

During development of BBB *Trojan horses*, validating the results at some stage in an *in vivo* model is still essential to understand systemic complexity and off pathways interactions. The use of animal models could however be minimised by utilisation of reproduceable *in vitro* models. Here, a first step has been taken in creating a transwell assay based on a membrane of functionalised nanofibrillar recombinant spider silk. Even if further optimisation is needed, and validation of the translatability to *in vivo* models is essential, the results are so far promising. Several further developments can be envisioned to improve the formation of tighter membranes such as, introducing flow,\cite{296} retinoic acid,\cite{297} or co-culturing of astrocytes which trigger tight junction formation between endothelial cells within the BBB.\cite{116}, \cite{117} The model shows potential for screening of BBB transcytosis for biomolecules in early discovery phase. Furthermore, the flow cytometry assay is a relatively straightforward way of screening for endocytosis, which could be used to identify the most promising candidates prior to the transcytosis assay. The recombinant silk has some structural similarities to ECM\cite{276} and a comparison study with respect to cell differentiation between cell lines grown in this model and *in vivo* tissue by for example transcriptomics would be interesting. This could help in further validating the translatability between models and *in vivo* settings. Moreover, adapting the assay for human hCMEC/D3 brain endothelial cells would be an obvious next step. Even if further optimisation is needed, and validation of the translatability to *in vivo* models is essential, small steps are a start.

As shown in the fifth paper, selection from affinity protein libraries towards a target is today often successful in terms of generating binders, but achieving the appropriate biological response is many times as important and might be more challenging. With the possibility to easily screen more variants from the selection outputs, the probability of finding a variant that yields a biological response may be increased. With the use of *in vitro* models and potential automation in the future, the higher throughput would allow for
characterisation of more candidates, which will aid in drug development in the early discovery phase.

With the recent advances towards earlier and more reliable diagnostics, by for example discovery of new biomarkers and development of functional MRI, the classification of different NDs is improving. An important part of treating NDs is the time of diagnosis. Early and correct diagnosis of the disease will be important as preventative and early treatment is likely more beneficial since regrowth of neurons of the brain is slow and limited. Moreover, inhibiting the disease progression before the cognitive decline has started would remarkably improve the patient’s quality of life.

Further, the general understanding of the disease mechanisms and correlations between genetics, environmental triggers, and the immune response is broadening the knowledge of this disease area. With increased knowledge, leading to novel treatment options and public enlightenment of common risk factors for the many sporadic cases, the negative trend of increased prevalence and mortality of NDs might finally be turned around. Several novel DMTs are in preclinical and clinical trials and better understanding about the most effective treatment strategies will unfold. As with cancer, one treatment is likely not the answer for all patients and might need to be adapted to each patient. Especially since several NDs can be progressing simultaneously and many subtypes of each disorder exist. Repurposing drugs between different disorders is a probable future, once options exist. In addition, when more approved drugs are available, treatment combinations will hopefully result in a leap in efficacy, similar to what has happened in oncology. However, with all uncertainty that surrounds NDs today, many clinical trials will likely follow with inadequate treatment response in the near future. Hopefully, with more positive results (than negative), as recently with the Aducanumab and Lecanemab approvals, one will continue to learn about the clinical effects, disease progressions, and continue trying to advance research around the newly discovered pathways and next-generation drugs.
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