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Synthesis and characterization of fluorescent stilbene-based probes targeting amyloid fibrils

Jun Zhang

Division of Chemistry
Department of Physics, Chemistry and Biology
Linköping University, Sweden

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During the course of the research underlying this thesis, Jun Zhang was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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Abstract

Alzheimer's disease (AD) is characterized by two main protein aggregate hallmarks in the brain: extracellular deposition of the amyloid- β ($A\beta$) in senile plaques and intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein. The past decade has seen great progress in the development of imaging probes for the non-invasive detection of $A\beta$ and tau aggregates. Here positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI), are highly promising technologies for clinical diagnostics. However, as a research tool, optical imaging is superior because it is real-time, sensitive, inexpensive, not radioactive and that it in particular affords high-resolution studies both *in vitro* and *in vivo*. Fluorescent probes are especially useful for designing novel binding scaffolds for structure investigations of protein aggregates. This thesis describes design, synthesis and evaluation of a series of fluorescent probes for detection of amyloid fibrils, especially $A\beta$ or tau aggregates *in vitro*.

Firstly, *trans*-stilbenoid vinylbenzene-1,2-diol with benzene, naphthalene, anthracene, and pyrene are investigated with respect to their photophysical properties free in solution and when bound to amyloid fibrils, including time-resolved fluorescence measurements. It is noted that the extended conjugated systems retained the amyloid targeting properties of the probes and both the anthracene and pyrene moieties extensively enhanced the fluorescence intensity and prolonged lifetimes.

Secondly, the synthesis of two molecules, Py1SA and Py2SA, based on pyrene linked to salicylic acid via a *trans*-stilbene C = C bond is presented. The compounds show strikingly different emission spectra when bound to preformed $A\beta$ 1-42 fibrils as well as to fibrils from four other distinct proteins. Additionally, excited state intramolecular proton transfer (ESIPT) coupled-charge transfer (ICT) is observed for the anionic form of the probes in polar solvents. This is likely the reason for the spectral differences of the probes when bound to amyloid fibrils.

Moreover, the synthesis of a further development of the Congo red analogue X-34 [2,5-bis(4'-hydroxy-3'-carboxy-styryl) benzene] by rational

design and synthesis is described. Full photophysical characterization was performed, including recording absorbance and fluorescence spectra, Stokes shift, quantum yield and fluorescence lifetimes. All ligands displayed high affinity towards recombinant amyloid fibrils of A β 1-42 and tau as well as selectivity towards the corresponding disease-associated protein aggregates in human post mortem AD tissue.

Lastly, the synthesis of a set of 2,1,3-benzothiadiazole (BTD)-based ligands with different conjugated spacers and variable patterns of OH substitutions of bis-styryl-BTD prototypes were developed. A β binding affinities (A β 1-42 and A β 1-40 fibrils) and the specificity towards A β plaques of all ligands were determined. These findings extend the structure to activity relationships of BTD-based ligands for A β fibril binding.

Throughout the studies in this dissertation, new interesting properties of small molecule fluorescence probes have been discovered and analyzed. This knowledge should facilitate the development of noninvasive probes for early detection of Alzheimer's disease and to distinguish different A β fibril polymorphs.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom definieras av två i hjärnan tydliga kännetecken: extracellulär avlagring av amyloid- β ($A\beta$) i senila plack och intracellulära neurofibrillära trassel (NFTs) som består av hyperfosforylerat tau-protein. Under det senaste decenniet har stora framsteg skett i utvecklingen av medicinsk avbildning för icke-invasiv kvantifiering av $A\beta$ och tau aggregat. Här är positron emissions tomografi (PET), single-photon emission computed tomography (SPECT) och magnetisk resonans avbildning (MRI), mycket lovande tekniker för klinisk diagnostik. Som ett forskningsverktyg är däremot optisk avbildning överlägsen eftersom den sker i realtid, är känslig, billig, icke-radioaktiv och att den i synnerhet tillåter högupplösta studier både *in vitro* och *in vivo*. Molekylära fluorescensprober är speciellt användbara för utprovning av olika bindningsställen för strukturundersökningar av proteinaggregat. Denna avhandling beskriver en serie syntetiska fluorescerande prober för detektering av $A\beta$ eller tau-aggregat *in vitro*.

Trans-stilbenoid vinylbensen-1,2-diol med bensen, naftalen, antracen och pyren undersöktes med avseende på deras fotofysikaliska egenskaper fritt i lösning och när de var bundna till amyloidfibriller. Detta inkluderade tidsupplösta fluorescensmätningar. Det visades att de utvidgade konjugerade systemen fortsatt behöll probens amyloidbindande egenskaper och att både antracen- och pyrenderivaten förbättrade i hög grad fluorescensintensiteten och förlängde livstiden för fluorescensen för proverna bundna till amyloida fibriller.

För det andra presenteras syntesen av två nya molekyler, Py1SA och Py2SA, baserade på pyren kopplad till salicylsyra via en trans-stilben C = C-bindning. Dessa prober visade påfallande olika emissionsspektra när de band till $A\beta$ 1-42 fibriller såväl som till fibriller från fyra andra distinkta proteiner. Denna stora skillnad i fluorescensspektra härrör sannolikt från intramolekylär protonöverföring (ESIPT) kopplad till laddningsöverföring (ICT) för den anjoniska formen av proverna. Denna hypotes stärktes av studier av proverna i polära lösningsmedel.

Dessutom beskrivs syntesen av en samling derivat av X-34 [2,5-bis (4'-hydroxi-3'-karboxi-styryl) bensen]. X-34 är analog till amyloidproben Kongo röd. Full fotofysikalisk karakterisering utfördes vilket innefattande absorbans- och fluorescensspektra, Stokes shift, kvantutbyte och fluorescenslivstider. Alla ligander uppvisade hög affinitet gentemot rekombinanta amyloidfibriller av A β 1-42 och tau såväl som selektivitet mot motsvarande sjukdomsassocierade proteinaggregat i vävnad från Alzheimers sjukdom.

Slutligen utvecklades syntesen av en uppsättning 2,1,3-bensotiadiazol (BTD)-baserade prober med olika konjugerade system och med variabla mönster av OH-substitutioner av bis-styryl-BTD-derivat. A β -bindningsaffiniteter (A β 1-42 och A β 1-40 fibriller) och specificiteten mot A β -plack av samtliga prober bestämdes. Dessa fynd utökar kunskapen av strukturaktivitets-samband för BTD-baserade ligander för A β -fibril bindning.

Genomgående för studierna i denna avhandling så har nya intressanta egenskaper hos småmolekylära fluorescensprober påvisats och analyserats. Denna kunskap bör underlätta utvecklingen av icke-invasiva prober för tidig detektion av Alzheimers sjukdom och för att särskilja olika A β -fibrilpolymorfer.

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Papers included in the thesis

- I. ***Trans*-stilbenoids with Extended Fluorescence Lifetimes for Characterization of Amyloid Fibrils**
J. Zhang, A. Sandberg, X. Y. Wu, S. Nyström, M. Lindgren, P. Konradsson and P. Hammarström*
ACS Omega 2017, 2, 4693.
- II. **Intramolecular proton and charge transfer of pyrene-based trans-stilbene salicylic acids applied to detection of aggregated proteins**
J. Zhang, J. Wang, A. Sandberg, X. Y. Wu, S. Nyström, H. LeVine III, P. Konradsson, P. Hammarström, B. Durbeej and M. Lindgren*
Chemphyschem 2018, 19, 1 (Cover Feature).
- III. **Detection and imaging of A β 1-42 and Tau fibrils by re-designed fluorescent X-34 analogues**
J. Zhang, A. Sandberg, A. Konsmo, X. Y. Wu, S. Nyström, K. P. R. Nilsson, P. Konradsson, H. LeVine III, M. Lindgren and P. Hammarström*
Chem. Eur. J. 2018, 24, 7210.
- IV. **Phenolic bis-styrylbenzo[c]-1,2,5-thiadiazoles as probes for fluorescence microscope mapping of A β plaque heterogeneity**
J. Zhang, A. Konsmo, A. Sandberg, X. Y. Wu, S. Nyström, U. Obermüller, B. M. Wegenast-Braun, P. Konradsson, M. Lindgren and P. Hammarström*
J. Med. Chem. 2018 (submitted).

Contribution to included papers

- I. Jun Zhang (JZ) participated in the planning of the project, performed all the experiments, except the purification of proteins and the preparation of corresponding fibrils. JZ analysed the results and wrote the manuscript draft.
- II. JZ participated in the planning of the project, performed all the experiments, except the purification of proteins and the preparation of corresponding fibrils as well as EC₅₀ assay. JZ analysed the results and wrote the manuscript draft.
- III. JZ participated in the planning of the project, performed all the experiments, except the purification of proteins and the preparation of corresponding fibrils as well as EC₅₀ assay. JZ analysed the results and wrote the manuscript draft.
- IV. JZ participated in the planning of the project, performed all the experiments, except the purification of proteins and the preparation of corresponding fibrils as well as lifetime and quantum yield assays. JZ analysed the results and wrote the manuscript draft.

Conference contributions

- ❖ 1st National Meeting of Swedish Chemical Society. Jun. 17-20 2018, Lund Sweden
[Poster] **Zhang J.**, Sandberg A., Nyström S., Wu X. Y., Konradsson P., Lindgren M. and Hammarström P. Synthesis and characterization of phenolic bis-styryl-benzo [c]-1,2,5-thiadiazoles as long-wavelength probes for imaging of A β aggregates in Alzheimer's disease.
- ❖ 6th Amyloid Disease Annual Meeting - Molecular Perspectives of Misfolded Proteins. Sep. 06-08 2017, Norrköping Sweden
[Poster] **Zhang J.**, Sandberg A., Wu X. Y., Nyström S., Lindgren M., Konradsson P., and Hammarström P. Fluorescent derivatives of Congo red for the detection of A β and Tau aggregates.
- ❖ 5th Global Chemistry Congress. Sep. 04-06 2017, London UK
[Poster] **Zhang J.**, Sandberg A., Wu X. Y., Nyström S., Lindgren M., Konradsson P., and Hammarström P. Fluorescent derivatives of Congo red for the detection of A β and Tau aggregates.

Thesis Committee

SUPERVISOR

Peter Konradsson, Professor

Division of Chemistry, Department of Physics, Biology and Chemistry
Linköping University, Sweden

CO-SUPERVISOR

Per Hammarström, Professor

Division of Chemistry, Department of Physics, Biology and Chemistry
Linköping University, Sweden

Xiongyu Wu, Research Engineer

Division of Chemistry, Department of Physics, Biology and Chemistry
Linköping University, Sweden

FACULTY OPPONENT

Lin Li, Professor

Institute of Advanced Materials
Nanjing Tech University, China

COMMITTEE BOARD

Anja Sandström, Associate Professor

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Uppsala University, Sweden

Malin Wennström, Associate Professor

Department of Clinical Sciences
Lund University, Sweden

Martin Ingelsson, Professor

Department of Public Health and Caring Sciences
Uppsala University, Sweden

Abbreviations

AD	Alzheimer's disease
A β	Amyloid beta fragment from amyloid- β precursor protein
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BINAP	2,2'-bis(diphenylphosphino)-1,1'-binaphthyl
BODIPYs	Boron-dipyrromethenes
BTB	2,1,3-benzothiadiazole
DCM	Dichloromethane
DMF	<i>N, N</i> -Dimethylformamide
DMSO	Dimethyl Sulfoxide
EA	Ethyl acetate
ESIPT	Excited state intramolecular proton transfer
FDA	Food and Drug Administration
GSIPT	Ground state intramolecular proton transfer
3,2-HNA	3-hydroxy-2-naphthoic acid
HOMO	Highest occupied molecular orbital
ICT	Intramolecular charge transfer
Ins.	Insulin
IMHB	Intramolecular hydrogen bond
LUMO	Lowest unoccupied molecular orbital
Lys.	Lysozyme
MRI	Magnetic resonance imaging
MS	Methyl salicylate
NFT	Neurofibrillary tangle
NiCl ₂ (dppf)	[1,1'-bis(diphenylphosphino)ferrocene]dichloronickel(II)
NIRF	Near-infrared fluorescence
NMDA	N-methyl-D-aspartate receptor
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PHOX	Phosphinooxazolines
PrP.	Prion protein
Pd(dppe)Cl ₂	[1,2-Bis(diphenylphosphino)ethane]dichloropalladium(II)

Pd(dppf)Cl ₂	[1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II)
Pd(PPh ₃) ₄	Tetrakis(triphenylphosphine)palladium
Pd(PPh ₃) ₂ Cl ₂	Bis(triphenylphosphine)palladium(II) dichloride
PT	Proton transfer
SA	Salicylic acid
SAR	Structure-activity relationship
SPECT	Single-photon emission computed tomography
SSNMR	Solid state nuclear magnetic resonance
Tau	Microtubule associated protein tau
TCSPC	Time-correlated single-photon counting
TLC	Thin-layer chromatography
TTR	Transthyretin
ThT	Thioflavin T

Table of Contents

Preface	1
1. Introduction.....	2
1.1. Palladium-catalyzed coupling reactions.....	2
1.1.1. <i>Heck reaction</i>	2
1.1.2. <i>Sonogashira reaction</i>	3
1.1.3. <i>Suzuki reaction</i>	5
1.2. Excited state intramolecular proton transfer (ESIPT)	7
1.3. 2,1,3-Benzothiadiazole (BTD) derivatives as fluorescent probes: beyond classical scaffolds	8
1.4. Amyloid fibrils	9
1.4.1. <i>Definition of amyloid</i>	10
1.4.2. <i>Amyloid structure</i>	11
1.4.3. <i>Amyloid formation</i>	13
1.5. Alzheimer's disease	15
1.5.1. <i>Amyloid-β cascade hypothesis</i>	15
1.5.2. <i>Tau cascade hypothesis</i>	17
1.6. Amyloid ligands	18
1.6.1. <i>Pan-amyloid probes</i>	19
1.6.2. <i>Aβ fibril fluorescent probes</i>	21
1.6.3. <i>Tau fibril fluorescent probes</i>	30
2. Research aim	37
3. Methodology.....	38
3.1. Fluorescence spectroscopy	39
3.2. Fluorescence microscopy.....	40
3.3. Time-correlated single-photon counting (TCSPC)	41
3.4. Determination of fluorescence quantum yield	42
3.5. Dissociation constant (K_d) by fluorescence spectroscopy	43
4. Summary of papers.....	44
4.1. Paper I.....	44
4.2. Paper II	49

4.3. Paper III	54
4.4. Paper IV	58
5. Conclusions.....	64
6. Future Perspectives	65
7. References	66

Preface

Several years ago, my grandma was diagnosed with Alzheimer's disease (AD) and I saw all of the symptoms in different stages until her death. Then an interest for neurological diseases, especially AD, in general was born. During this period, my major, as a master student, is medicinal chemistry focusing on asymmetric synthesis in Sichuan University (China). It always appeared in my mind whether I can combine organic synthesis and neurological diseases. Then I, as a PhD student, have a chance to study focusing on organic synthesis under the direction of Peter Konradsson in Linköping University supported by China Scholarship-Council (CSC). And I know that my co-supervisor is interested in protein misfolding, amyloid formation and disease, both on the molecular level and in the cellular perspective. Then I have my own research topic: synthesis and characterization of fluorescent stilbene-based probes for detection and imaging of disease-associated protein aggregates.

This thesis summarized the results obtained during my PhD studies. The first part of the thesis is to give the reader a general introduction of this field and the current findings, as well as the methods used for my research. Next, a brief summary of the conclusions and findings from my papers can be found. Finally, future perspectives are discussed.

After four years training as a PhD student, I found myself particularly interested in this field and want to further pursue this area for postdoctoral training. I hope this thesis emphasizes the importance and significances of current research, and will increase your knowledge within the Alzheimer's field.

Hope you will enjoy reading my thesis!

Yours sincerely,

Linköping, September 2018

1. Introduction

1.1. Palladium-catalyzed coupling reactions

Palladium (Pd)-catalyzed coupling reactions consist of a family of cross-coupling reactions with different substrates: Kumada, Heck, Sonogashira, Negishi, Suzuki reaction et al. and have become one of the most widely applied methodology for C-C bond construction.¹ Due to the great successes and significance, the 2010 Nobel prize in chemistry was awarded to Richard Heck, Ei-ichi Negishi and Akira Suzuki for their roles in "palladium-catalyzed cross-couplings in organic synthesis". All the synthesized fluorescent probes in this thesis mainly involve Heck, Sonogashira and Suzuki reactions, as summarized as follows.

1.1.1. Heck reaction

The Heck reaction (also called the Heck-Mizoroki reaction) was discovered independently by Mizoroki in 1971 and Heck in 1972, and is generally referred to as the Pd-catalyzed reaction of an alkene with an unsaturated halide (or triflate) to give a substituted alkene, as shown in Scheme 1.1A.² The alkene, containing at least one hydrogen, is often electron-deficient such as an acrylate ester, and the halide (Cl, Br, I) or triflate is an aryl, or vinyl compound. Pd is usually the preferred metal as it tolerates a wide variety of functional groups such as carbonyl and hydroxyl groups, and commonly used Pd-catalysts are palladium(II) acetate, tetrakis (triphenylphosphine) palladium [Pd(PPh₃)₄] or palladium chloride and the like. The commonly used ligands are phosphine ligands such as triphenylphosphine, 2,2' -bis(diphenylphosphino)-1,1' -binaphthyl (BINAP) or phosphinooxazolines (PHOX). The commonly used bases include potassium carbonate, sodium acetate or triethylamine.³⁻⁴

The Heck reaction mechanism, based on the Pd catalyst, includes 4 steps (Scheme 1.1B).⁵

- ❖ Step A: oxidative addition in which Pd inserts itself into the aryl-X bond to form a tetra-substituted complex II.

- ❖ Step B: syn insertion in which the alkene inserts itself into the Pd-C bond to form complex III.
- ❖ Step C: β -hydride elimination with the formation of a target compound.
- ❖ Step D: reductive elimination in which the Pd(0) compound is re-generated by the addition of base.

Scheme 1.1 A) General Heck-type reaction. B) General catalytic cycle.⁵

1.1.2. Sonogashira reaction

The Sonogashira reaction (also called the Sonogashira-Hagihara reaction) is a Pd and copper(I) catalyzed cross-coupling reaction of a terminal alkyne and an aryl or vinyl halide to give a substituted alkyne, as presented in Scheme 1.2A.⁶ The most common catalysts used are bis(triphenylphosphine)palladium(II) dichloride $[\text{Pd}(\text{PPh}_3)_2\text{Cl}_2]$ and $\text{Pd}(\text{PPh}_3)_4$. $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ is more soluble in water and stable than $\text{Pd}(\text{PPh}_3)_4$, both catalysts require up to 5% loading to afford good yield. In addition to this, bidentate ligands have also been used, such as [1,2-bis(diphenylphosphino)ethane]dichloropalladium(II) $[\text{Pd}(\text{dppe})\text{Cl}_2]$ and [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) $[\text{Pd}(\text{dppf})\text{Cl}_2]$.

The copper(I) catalyst is usually a copper(I) salt, such as copper iodide. The reaction mechanism is not clearly understood but revolves around a Pd cycle and a copper cycle (Scheme 1.2B).⁷

The Pd cycle includes four steps:

- ❖ Oxidative addition in which the active Pd catalyst $L_2Pd(0)$, complex I (14 electron compound) inserts itself into the aryl or vinyl-X to produce a Pd(II) intermediate, complex II. This step is believed to be the rate-limiting step of the reaction.
- ❖ *Trans*-metalation in which complex II reacts with the copper acetylide produced in the copper cycle to yield complex III, expelling the copper halide.
- ❖ *Trans-cis* isomerization in which both organic ligands are *trans*-oriented and converted to *cis* to produce complex IV.
- ❖ Reductive elimination as the final step in which complex IV is reduced to produce the alkyne with regeneration of the Pd catalyst.

The copper cycle consists of two steps:

- ❖ The presence of base results in the formation of a π -alkyne complex, which further makes the terminal proton on the alkyne more acidic, leading to the formation of copper acetylide.
- ❖ Then the copper acetylide reacts with the palladium intermediate II via *trans*-metalation with regeneration of the copper halide.

Scheme 1.2 A) General Sonogashira-type reaction. B) General catalytic cycle.⁷

1.1.3. Suzuki reaction

The Suzuki reaction (also called Suzuki–Miyaura reaction or Suzuki coupling) is referred to as the reaction of an organoboron and an organohalide or triflate catalyzed by a Pd(0) complex (Scheme 1.3A), which was first published in 1979 by Akira Suzuki.⁸ Organoboranes include aryl boronic acids and aryl-trifluoroborate salts. The Pd-based catalysts have been the most frequently used ones, like $\text{Pd}(\text{PPh}_3)_4$, although nickel catalysts have also been used such as [1,1'-bis(diphenylphosphino)ferrocene]dichloronickel(II) $[\text{NiCl}_2(\text{dppf})]$.⁹ Being able to use relatively cheap and easily prepared reagents, especially using water as a solvent, makes this reaction more economical and practical.

From the perspective of Pd catalyst, the Suzuki reaction mechanism contains three basic steps: oxidative addition, *trans*-metalation, and reductive elimination as demonstrated in Scheme 1.3B.⁸

- ❖ **Oxidative addition:** complex I reacts with the aryl or vinyl halide to yield complex II, which is a Pd(II) intermediate. The oxidative addition is the rate determining step in the catalytic cycle.
- ❖ **Trans-metalation:** the reaction of complex III with an organoborane compound produced by the reaction of boronic acid with base via trans-metalation, produces a complex IV in the catalytic cycle.
- ❖ **Reductive elimination:** this step gives the desired coupling product and regenerates the Pd(0) complex for the catalyst under basic conditions.

Base is significantly important for Suzuki reaction as investigated by many chemists. Among them, Duc and coworkers found that the base has three functions: 1) forming the palladium complex III; 2) producing the trialkyl borate; 3) the acceleration of the reductive elimination.¹⁰

Scheme 1.3 A) General Suzuki-type reaction. B) General catalytic cycle.⁸

1.2. Excited state intramolecular proton transfer (ESIPT)

In 1956, Weller firstly introduced the field of photochemistry to a new area of research through experiments with salicylic acid (SA) and methyl salicylate (MS), namely ESIPT reaction.¹¹⁻¹² As displayed in Scheme 1.4, SA mainly exists as R-form and E-form in solutions, and the latter is the dominant form because of the stronger intramolecular hydrogen bond. R-form cannot undergo excited-state tautomerization, since it involves a weak $\text{O-H}\cdots\text{O-H}$ bond between the acidic phenolic OH and the oxygen atom of the carboxylic OH and is responsible for the UV band. The E-form can undergo excited-state tautomerization because it involves a strong $\text{O-H}\cdots\text{O}=\text{C}$ bond between the acidic phenolic OH and the oxygen atom of the carboxylic $\text{C}=\text{O}$ and the large Stokes shifted fluorescence is assigned to the formation of the K-form*, resulting from ESIPT. Following the pioneering work of Weller, a great deal of research based on SA and its relatively simple derivatives has been directed to the study of photoinduced proton transfer (PT) process across intramolecular hydrogen bonds (IMHBs). Among the studies, the ESIPT is more facile in the neutral form than in the anion of SA and its derivatives. However, few examples do exist. Mishra et al. has reported that ESIPT is observed only in the anionic form of 3-hydroxy-2-naphthoic acid by experimental and theoretical investigations.¹³ In addition, quite a few reports have appeared in literature on the study of substitution effect on ESIPT of SA.¹⁴⁻¹⁵

Scheme 1.4 A) Theory of the ground- and excited-state species of SA as well as a schematic of its photo-physics. B) Different species of SA. GS IPT = ground state intramolecular proton transfer.

1.3. 2,1,3-Benzothiadiazole (BTD) derivatives as fluorescent probes: beyond classical scaffolds

To date, Coumarins, fluoresceins, rhodamines, boron–dipyrromethenes (BODIPYs), cyanines, and phenoxazines are among the most used fluorescent derivatives.¹⁶ Meantime, the limitations associated within them are already well-known.¹⁶ 2,1,3-benzothiadiazole (BTD) derivatives, as small fluorescent molecules, are a new class of bio-probes with attractive photophysical properties (see Figure 1.1A): high molar extinction coefficients, large Stokes shifts, high quantum yields, high storage stability, bright emission, good signal-to-noise ratios and low fading after long periods of irradiation as well as easy to pass the cell membranes.¹⁷ Although a large number of fluorescent small molecule BTD derivatives were applied to bioimaging analyses of several cell types, little is known about this class of bio-probes used to stain A β plaques and NFTs in Alzheimer’s disease (AD). Nilsson and coworkers reported on L3 (Figure 1.1B),

which can intensely stain A β plaques,¹⁸ and HS-169 (Figure 1.1B) intensely stain A β plaques and NFTs.¹⁹ In view of all the positive effects of the photoluminescent properties of π -extended BTDs, we wish this new class of bio-probes could fill the gap not occupied by the use of current classical scaffolds and be key player in the future of molecular bio-probes, especially in the field of AD.

Figure 1.1 A) Chemical structure of BTB derivatives and their attractive photo-physical properties.¹⁷ Note that R₂ and R₃ are usually H atoms and that R₁ and R₄ are the groups in the positions commonly used for π -extensions. B) Chemical structures of BTB derivatives reported previously.¹⁸⁻¹⁹

1.4. Amyloid fibrils

Proteins, as large biomolecules, control and regulate essentially every chemical process required for life, such as responding to stimuli, DNA replication, catalyzing metabolic reactions and transporting molecules from one location to another. Each protein is composed of amino acids that are joined by consecutive peptide bonds forming long polymeric chains. Under physiological conditions, these strings of amino acids fold in a specific manner to adopt a particular three-dimensional structure. This structure, referred to as the native state, is necessary for the protein to perform its function. The cell has evolved a range of auxiliary systems

to assist in the protein folding process but these systems might get overwhelmed during pathologic conditions resulting in the assembly of misfolded proteins. For a certain class of proteins, this situation causes them to aggregate and give rise to deposits of highly organized fibrillar material referred to as amyloid, leading to various human diseases, for example, A β peptides in Alzheimer's disease (AD, introduced in the following sections), α -synuclein in Parkinson's disease and the prion protein in Creutzfeldt-Jakob disease.²⁰

1.4.1. Definition of amyloid

The term, amyloid, derived from the Latin amyllum and the Greek amylon, comes from the early mistaken identification in 1854 by the German physician scientist Rudolph Virchow of the substance as starch, based on crude iodine-staining techniques.²¹ For a period, the scientific community debated whether or not amyloid deposits are fatty deposits or carbohydrate deposits until it was found (in 1859) that they are, in fact, deposits of proteinaceous material.²¹

- ❖ The classical, histopathological definition of amyloid according to the Nomenclature Committee of the International Society of Amyloidosis is that amyloid is an extracellular, proteinaceous deposit exhibiting β -sheet structure perpendicular to the fibril axis. identified by apple-green birefringence when stained with Congo red and seen under polarized light.²² These deposits often recruit various sugars and other components such as Serum Amyloid P component, resulting in complex, and sometimes inhomogeneous structures.²³ Recently this definition has come into questions due to some classic amyloid species have been observed in distinctly intracellular locations.²⁴
- ❖ A more recent biophysical definition is broader, including any polypeptide that polymerizes to form a cross- β structure, *in vivo* or *in vitro*. Some of these, although demonstrably cross- β sheet, do not show some classic histopathological characteristics such as the Congo-red birefringence. Microbiologists and biophysicists

have largely adopted this definition,²⁵⁻²⁶ leading to some conflicts in the biological community.

1.4.2. Amyloid structure

Amyloid fibrils are insoluble and heterogeneous, so commonly used methods of structure determination of soluble proteins are difficult to apply on amyloids. Therefore, most studies have involved electron microscopy,²⁷ X-ray fiber diffraction,²⁸⁻²⁹ and more recently, solid state nuclear magnetic resonance (SSNMR)³⁰ and electro-paramagnetic resonance.³¹⁻³²

Electron and atomic force microscopy have shown that fibrils are straight, long and unbranched (Figure 1.2A) ranging in width from 60 to 130 Å with indefinite length³³ and usually consist of individual subunits named "protofilaments".³⁴⁻³⁶ These may vary in number and are often observed to twist around one another to form the mature fibril.^{34, 36-38} Synthetic amyloid-like fibrils, may vary in morphology and this may depend upon the assembly conditions.³⁶ Further cryo-electron microscopy studies demonstrated that synthetic amyloid fibrils formed by lysozyme,³⁸ insulin,³⁷ and A β (1-42)³⁹ are also composed of several protofilaments wound around one another. The numbers of protofilaments can differ from 2 to 6.³⁷

X-ray diffraction analysis has revealed that amyloid fibrils is "cross- β " (Figure 1.2B), sharing a particular core structure consisting of β -sheet conformation in which the NH and CO hydrogen bonding direction runs parallel to the fiber axis and perpendicular to the β -strands, much like the rungs of a ladder. In addition, the spacing between adjacent β -strands is approximately 4.7 Å, corresponding well with the standard length of hydrogen bonds between NH and CO groups of β -sheets, arranging in a parallel or antiparallel. The β -sheets are separated by approximately 10 Å.^{27, 40-41} More detailed X-ray diffraction patterns have been obtained from synthetic amyloid fibrils and additional information obtained has enabled molecular models to be proposed.⁴² In general, these models are cross- β in nature and the core of the structure is β -sheet conformation. The β -sheet ribbons are associated via side chain interactions that serve to stabilize the structure.⁴² So individual protomers are folded as a β -arch.

Figure 1.2 A β fibrils *in vitro*. A) electron micrograph revealing straight, long, unbranching fibrils. B) X-ray micrograph revealing the characteristic “cross- β ” diffraction pattern. C) SSNMR structural studies revealing the top view of the fiber with side chains (top) and as a carbon (middle) as well as showing the side view (down). D) Cross-sections of molecular models demonstrating polymorphism of A β 1-42 fibrils. (a, b) Two structures were almost identical; (c) whilst a different peptide fold and protofilament structure was observed with a third sample of A β 1-42 fibrils assembled *in vitro* and (d) combining cryo-EM and NMR in a recent study. Structures were generated in PyMol using the PDB codes: (a) 5KK3 (b) 2NAO (c) 5AEF (d) 5OVQ. (A-C) reproduced with permission from Prion, 2008, 2, 112-117. And (D) reproduced with permission from J. Intern. Med., 2018, 283, 218-237.

Recently, SSNMR structural studies have significantly gained our knowledge of the molecular structure of amyloid fibrils⁴³ and this work has been complemented by amyloid formed by a number of different disease associated peptides.³² SSNMR studies of A β fibrils associated with AD have shown that β -sheet, depending on the properties of the precursor polypeptide, could be arranged parallel or anti-parallel within the protofilaments.⁴⁴⁻⁴⁶ A β peptide folds into a β -arch structure that then associates with other molecules to form parallel, in register β -structure (Figure 1.2C).⁴⁷⁻⁴⁸ In addition, over the past 10-15 years, it has been acknowledged that amyloid fibrils are generally polymorphic at the molecular level.⁴⁹⁻⁵⁰ For instance, SSNMR or electron cryo-microscopy (cryo-EM) studies recently provided that A β 1-42 peptide can attain variable stable folds and protofilament structures (Figure 1.2D).⁵¹

1.4.3. Amyloid formation

In order for the protein to perform its natural task, the newly synthesized polypeptide chain needs to fold in a specific manner to adopt a particular three-dimensional structure, referred to as the native state. However, when a natively soluble and functional protein becomes partly unfolded or misfolded due to mutations, changes in the environmental conditions or chemical modifications, the protein begins to rearrange into aggregate and form amyloid aggregates leading to the associated amyloid disease.⁵² Both processes can be described as a multidimensional energy landscape (Figure 1.3) with Gibb's free energy on the y-axis and the width of the funnel representing entropy.⁵³⁻⁵⁴ Initially, the unfolded protein has a lot of conformational freedom but as the folding process proceeds, the molecule becomes more restricted giving rise to a conformational volume with the appearance of a funnel (green and red). Additionally, the amyloid state of protein can even be more stable. However, it is difficult to convert to the more stable amyloid state due to the high free energy barriers.⁵⁵

Figure 1.3 The protein folding energy landscape. The green area depicts the folding of native proteins and the red area depicts the misfolding of proteins. *Reproduced with permission from PhD thesis “Investigating Amyloid β toxicity in *Drosophila melanogaster*”, 2017, Maria Jonson.*

Amyloid fibrillation is known to be a nucleation-dependent process including three main phases: the lag, growth and stationary phases. In the lag phase, the native monomer is destabilized and undergoes a re-arrangement into β -sheet rich partly unfolded structures. Then the lag phase is continued by primary nucleation where interactions between the partly unfolded or misfolded monomers to form a nucleus.⁵⁶⁻⁵⁷ This process is thermodynamically disfavoured since the resulting intermolecular interactions do not outweigh the reduction in entropy and therefore considered as the rate-limiting step of the aggregation process. In addition, the length of the lag phase may be shortened and even abolished by the addition of pre-formed fibrillar species.⁵⁸ The growth phase begins as soon as the nucleus is established, forming fibrils which can catalyze further growth of soluble oligomers and pre-fibrillar structures, protofibrils. The stationary phase, in which mature fibrils are in assembly/disassembly equilibrium with the monomers, is reached.^{55, 59} It was reported that the nucleation rate together with the growth rate of a fibril might influence the age of onset for amyloid-related diseases.⁶⁰

Figure 1.4 Schematic of amyloid fibrillation of a protein.

1.5. Alzheimer's disease

AD is a chronic neurodegenerative disease that was first reported by the psychiatrist Alois Alzheimer from Germany in 1906.⁶¹ AD is characterized by features including memory impairment, disorientation, mood and behavior changes as well as difficulties in expressing yourself and understanding the spoken and written language.⁶² AD is an age-dependent disease, including three stages: early stage, middle stage and late stage, with a progressive pattern of cognitive and functional impairment.⁶³ In 2016 about 47 million people lived with AD in the world, and this number is continuously increasing, especially due to the increased life expectancy worldwide.⁶⁴ Usually, over 65 years of age in people begins with AD and about 6% of people 65 years and older worldwide is affected.⁶⁵ Hence AD is a global public health issue, demanding huge amounts of resources, especially in developed countries.⁶⁶

1.5.1. Amyloid- β cascade hypothesis

To date, the effect of AD on the brain is clear although the causes aren't yet fully understood. It is known that less than 5%, AD is caused by specific genetic changes.⁶⁷ Many hypotheses about AD have been developed,

including A β hypothesis,⁶⁸⁻⁶⁹ tau hypothesis,⁶⁹⁻⁷⁰ cholinergic hypothesis,⁷¹⁻⁷² and other hypotheses.⁷³⁻⁷⁵ The first two terms are the most dominant hypothesis in the field of AD pathogenesis.

In 1991, the amyloid cascade hypothesis was proposed by John Hardy and David Allsop that extracellular A β deposits are what initiates the cascade of events leading to pathological changes, neurodegeneration and cognitive decline in a linear pathway.⁶⁸⁻⁶⁹ The location of gene for amyloid precursor protein (APP), a transmembrane protein with a small intracellular domain and a larger extracellular domain, supports this assumption. As demonstrated in Figure 1.5, APP processing can occur in either the non-amyloidogenic pathway or the amyloidogenic pathway. Furthermore the hypothesis is strengthened by findings that a majority of mutations linked to early-onset AD, with the most pronounced being the Swedish mutation, acts by increasing the total amount of A β . The ratio of the fibrillogenic A β 1-42 peptide compared to other alloforms are increased for certain mutations in APP, and the levels of amyloid plaques are associated with familial mutations in the presenilin 1 gene coding for the catalytic subunit of the γ -secretase cleaving APP (β -CTF) within the membrane (Figure 1.5).⁷⁶⁻⁷⁷ However, an increasing number of studies show that the density of plaques in brain have a weak correlation with the degree of dementia, and that plaques in healthy individuals also occur.⁷⁸ Therefore, the theory has been modified as evidence providing a strong correlation between soluble A β oligomers and disease pathology.⁷⁹ Studies demonstrate that these toxic A β oligomers precede fibril formation and are responsible for the cascade involving amyloid deposition, inflammation, oxidation stress that in turn leads to clinical disease.⁸⁰

All these reactions suggest that A β is not sufficient to cause the complex pathology of AD and there are more to the story than A β alone.⁸¹⁻⁸² This, together with recent understanding of the physiological roles of A β is challenging the hypothesis and has revised the view on how to approach the development of therapies against AD.

Figure 1.5 The proteolytic processing of APP including the non-amyloidogenic pathway and amyloidogenic pathway.

1.5.2. Tau cascade hypothesis

Tau proteins, mostly associated with microtubules, are abundant in the central nervous system with predominate expression in the axonal compartment of neurons.⁸³ In normal adult human brain tau proteins exist in six isoforms, 0N3R, 1N3R, 2N3R, 0N4R, 1N4R and 2N4R, ranging from 352 to 441 amino acids (Figure 1.6A).⁸⁴ In AD where tau filaments are present, tau is hyperphosphorylated and the functional consequences of this have been under investigation for a long time. The tau hypothesis indicates that tau protein aggregation causes the disease cascade.⁶⁹ Hyperphosphorylated tau accumulates as paired helical and straight filaments and finally forms aggregated tau protein (also known as tau neurofibrillary tangles, NFTs) (Figure 1.6B).⁸⁴ The loss of microtubule-stabilizing tau protein leads to the degradation of the cell's cytoskeleton which collapses the neuron's transport system, resulting in malfunctions in biochemical communication between neurons and, ultimately the death of the cells.⁸⁵⁻⁸⁶

Figure 1.6 A) Six tau isoforms in adult human brain.⁸⁴ B) The formation process of NFTs.⁸⁴ (B) reproduced with permission from *Chem. Soc. Rev.* 47, 2249-2265.

1.6. Amyloid ligands

To date, the pathological mechanism of AD is elusive. Food and Drug Administration (FDA)-approved medications currently available including one N-methyl-D-aspartate receptor (NMDA) receptor antagonist and four acetylcholinesterase inhibitors, only provide temporary relief from symptoms. None of these treatments halts the progression of this terminal disease. This argues that more research is needed on early-stage diagnosis to further understand and treat this disorder.

The past decade has seen great progress in the aspect of imaging probes for the non-invasive detection of A β plaques and NFTs, mainly depending on techniques such as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI).⁸⁷⁻⁸⁹ Recently, optical imaging, especially near-infrared fluorescence (NIRF) imaging, when compared to PET, SPECT and

MRI, is highly promising because it is real-time, not radioactive, inexpensive and of high-resolution both *in vivo* and *ex vivo*.⁹⁰ Optical imaging is an imaging technique using ultraviolet, visible, and infrared light for imaging. Especially, NIRF probes are favorable for A β and Tau detection *in vivo* because emission wavelengths in the NIR range (650–900 nm) are distinct from the autofluorescence of biological matter, which is suitable for *in vivo* applications. In addition, for a NIRF imaging setup, when compared with PET, costs are much lower, the data analysis takes less time, and specialized personnel and labs are not needed.

In principle, an ideal fluorescent probe for imaging of A β plaques or tau tangles should have the following properties.⁹¹⁻⁹⁴

- ❖ Straightforward synthesis.
- ❖ High selectivity and binding affinity.
- ❖ High quantum yield and significant changes in the fluorescence properties upon binding to A β plaques or NFTs.
- ❖ A suitable wavelength of excitation and emission greater than 450 nm to minimize background fluorescence from brain tissue, ideally 650-900 nm for *in vivo* detection.
- ❖ High blood-brain barrier (BBB) permeability (logP values between 2 and 3.5, or clogP < 5.0 are considered optimal).⁹⁵
- ❖ High metabolic stability.
- ❖ Fast washout kinetics from normal brain regions.
- ❖ Low toxicity.

1.6.1. Pan-amyloid probes

There is a significant overlap between the characteristics and architectures of chemical probes for both A β plaques and tau tangles as well as other amyloids, since these targets share some striking similarities. For instance both exhibit β -sheet conformation and stacking at the core of

their structure and represent an environment that is significantly less hydrophilic than the surrounding medium. Initially, staining of amyloid in brain sections for pathological post-mortem analysis was performed using traditional tissue staining dyes such as Crystal violet, Hoffman's violet and Methyl green. Other probes used for this purpose are the phenothiazines including Toluidine blue. These dyes show a colour change when bound to amyloids, as compared to their staining of the surrounding tissue (Figure 1.7).

Thioflavin T and S (ThT and ThS, Figure 1.7) are prototypical molecular rotors, showing a dramatic increase in fluorescence when their rotational freedom is restricted. Owing to their flat structure, these probes are particularly well suited to bind to β -sheet rich aggregates. Therefore, they show significantly strong staining on A β plaques and tau tangles.

Congo Red (Figure 1.7) is commonly used for fluorescent staining of the plaques and tangles in post-mortem brain sections of AD patients.⁹⁶ Congo red binding resulting in green birefringence under crossed polarizers is a criterion of diagnosis of amyloid deposits.²⁰ Chrysamine-G (CG) (see structure in Figure 1.7), the first derivative of CR, was synthesized and used by Klunk *et al.* as an amyloid dye.⁹⁷⁻⁹⁸ This soon led to the synthesis of a number of bis-styrylbenes with strong binding affinity, which include X-34,⁹⁹ FSB,¹⁰⁰ BSB,¹⁰¹⁻¹⁰² ISB (see structures in Figure 1.7).¹⁰³ These compounds represent a more lipophilic version of CG to increase the potential for BBB penetration. However, neither of these compounds is selective for A β plaques or tau tangles, rather staining all of the proteinaceous aggregates in the brain. Klunk *et al.* further synthesized Methoxy-X04 (Figure 1.7) by removing all carboxylic acid groups, resulted in efficient BBB penetration and *in vivo* staining of the brain amyloid content.¹⁰⁴ Sharing a π -conjugated backbone of similar length, curcumin (Figure 1.7), a natural probe extracted from turmeric, has been shown to fluorescently stain various protein aggregates including A β and tau fibrils.

Figure 1.7 Chemical structures of the pan-amyloid probes.⁹⁶⁻¹⁰⁴

1.6.2. A β fibril fluorescent probes

Currently, the reported A β fibril fluorescent probes largely share a common push-pull structure and are highly sensitive to solvent polarity. They are essentially non-fluorescent in aqueous solutions, but highly fluorescent upon binding to A β fibrils or in nonpolar solvents. In this section, I will introduce the different scaffold features of this mode.

Thioflavin-T and its derivatives

The fluorescent probe ThT (Figure 1.7) has become the widely used "gold standard" for selective amyloid aggregates identification *in vitro* and *in vivo* since first introduced in 1959. Then a variety of derivatives of ThT have been developed during the past years. Although many of them are

radiolabelled for PET or SPECT imaging, there are still some probes that display high affinity for A β aggregates.

Compound **15** (BTA-1, Figure 1.8) is a neutral ThT derivative, which showed an excellent affinity ($K_d = 3$ nM) in *in vitro* binding assay using A β 1-40 fibrils. When [^{11}C] BTA-1 was injected into mice, it displayed an excellent brain penetration with an initial brain uptake at 2 min of 3.0% dose /organ.¹⁰⁵ Compound **16** (12a, Figure 1.8) is a benzothiazole Schiff-base and has a high affinity towards AD brain homogenates with a K_i value of 4.4 nM and this compound can label A β plaques of AD brain tissue.¹⁰⁶ Compound **17** (Figure 1.8), from Jung's group, showed a high affinity ($K_d = 3.3$ μM) for A β fibrils and a significant fluorescence increase (36.1-fold) as well as a Log P = 3.7 suitable for potential BBB penetration.¹⁰⁷ Kung et al. reported a ThT derivative, compound **18** (IMPY, Figure 1.8), displaying a much better binding affinity towards A β 1-40 fibrils *in vitro* ($K_i = 15 \pm 5$ nM) and selective A β plaque labelling on post-mortem AD brain sections.¹⁰⁸ However, the fluorescence spectra of the above-mentioned probes are still in a range that limits their potential use *in vivo*.

Ono et al. designed and synthesized two push-pull benzothiazole derivatives, compound **19** (PP-BTA-1, Figure 1.8) and **20** (PP-BTA-2, Figure 1.8), with either benzothiazole or styryl-benzothiazole as the highly polarized bridge.¹⁰⁹ Results of inhibition assays on the binding to A β aggregates indicated that **19** and **20** may occupy a binding site on A β aggregates similar to that of ThT with IC₅₀ values for **19** and **20** were 0.12 and 0.11 μM , respectively. In addition, **19** and **20** can clearly stain A β plaques in both transgenic mice and human AD brain sections.

Figure 1.8 Chemical structures of Thioflavin-T derivatives.¹⁰⁵⁻¹⁰⁹

Curcumin and its derivatives

In AD research, curcumin (Figure 1.7) has been reported to have the following properties: anti-oxidation, anti-A β aggregation, inhibition of β -secretase and acetylcholinesterase, and A β -induced inflammation *in vitro*.¹¹⁰ Cole et al. reported that curcumin can label amyloid plaques, like ThS in AD and transgenic mice brain sections, and crosses the BBB to bind to plaques *in vivo*.¹¹¹ However, due to its short emission wavelength, practical applications of curcumin *in vivo* are limited.

Moore and colleagues designed and synthesized a novel class of NIRF probes derived from curcumin. In this structure, a difluoroboronate moiety and two *p*-dimethylamino phenyl groups were integrated into the curcumin scaffold to form a donor-accepter-donor architecture. Among them, compound **21** (CRANAD-2, Figure 1.9) functions as a smart probe because of drastic fluorescence changes (70-fold fluorescence intensity increase and 90 nm hypochromic shift), a lifetime change, and quantum yield improvement upon binding to A β aggregates. This probe has a high affinity ($K_d = 38.0$ nM) for A β aggregates, a reasonable Log P = 3 and a weak albumin interaction. Because bulky analogs **22** (CRANAD-6, Figure 1.9) didn't show significant fluorescence change, it was proved that the binding site of **21** is stereo-hindered, likely to be the hydrophobic site containing the core fragment (KLVFF).¹¹² Compound **21** was capable to selectively stain of A β plaques in a brain section of APP-PS1 transgenic mouse.¹¹² In another report from the same group, **21** was used as non-conjugated FRET pair in combination with compound **23** (CRANAD-5, Figure 1.9) for differentiating A β monomers from higher aggregated A β species.¹¹³

The same group reported another probe, compound **24** (CRANAD-3, Figure 1.9) by replacing benzene with pyridine and dimethylamino with diethylamino groups, with subsequent removal of the difluoroboron bridge. The big difference from **21** was that it also interacted with soluble A β monomers and dimers, and displayed fluorescence signal change except for significant fluorescence property changes upon binding to A β aggregates.¹¹⁴

Recently, more new analogs of **21** were designed and synthesized as NIRF probes for detection of soluble and insoluble A β species and inhibition of copper-ion induced A β aggregation.¹¹² Zhang et al. studied the structural stereohindrance compatibility of A β species and designed and synthesized a compound **25** (CRANAD-58, Figure 1.9). Compound **25** displayed different fluorescence response for soluble (monomers, dimers and oligomers) and insoluble A β species. *In vitro* staining assay revealed **25** could specifically highlight A β plaques that co-stained with ThS. *In vivo* NIRF imaging indicated that **25** was capable of differentiating transgenic and wild-type mice at the age of 4 months.¹¹⁵ Another analog, compound **26** (CRANAD-17, Figure 1.9) containing two copper coordinating imidazoles, could compete and interfere with copper induced A β cross-linking. *In vitro* anti-crosslinking studies indicated that **26** could induce 68% more of A β monomers as compared with non-treated samples, suggesting potential usage as a theragnostic agent.¹¹²

Figure 1.9 Chemical structures of curcumin derivatives.¹¹²⁻¹¹⁵

BODIPY-derived probes

Boron-dipyrromethene, BODIPY, is a classical fluorescent probe applied in a variety of fields.¹¹⁶ Boens et al. reviewed the notable features of BODIPY, all contributing to the appeal of this structure as an important

tool in a variety of imaging applications.¹¹⁷ One et al. reported the first BODIPY-derived fluorescence/SPECT dual probe **27** (BODIPY-7, Figure 1.10) containing a conjugated thiophene-phenyl chain for A β fibril detection.¹¹⁸ **27** has a modest affinity ($K_i = 108$ nM) for A β aggregates and is able to detect A β plaques in *in vitro* staining of brain sections. The low BBB permeability, the short wavelengths of absorption/emission, at 606/613 nm, and the narrow Stokes shift restrict *in vivo* imaging applications.¹¹⁸ Then another BODIPY-derived probe, **28** (BAP-1, Figure 1.10) with a maximal emission wave length of 648 nm, displayed excellent efficacy both *in vitro* and *in vivo*. **28** showed a high affinity ($K_d = 44.1$ nM) for A β aggregates and clearly stained A β plaques in the brains of transgenic mice. Further studies indicated that **28** could clearly differentiate between 25-month-old wild-type and Tg2576 mice at 1 h post-injection and possesses good metabolic kinetics *in vivo*.¹¹⁹ In 2013, several BAP-1 analogs, **29**, **30**, **31** and **32** (BAP-2, BAP-3, BAP-4 and BAP-5, Figure 1.10), which contain most of the advantageous features of **28** but a better maximal emission wavelength, up to 700 nm, were disclosed.¹²⁰ The measured K_d values for **29**, **30**, **31**, and **32** were 55, 149, 27, and 18 nM, respectively, suggesting a high affinity for A β 1–42 aggregates. Similar to **28**, they could selectively label A β plaques *in vitro*. However, the probe, **29**, was selected for *in vivo* imaging but failed due to the same problem of higher accumulation in the scalp than in the brain. This result indicates that appropriate structural modifications to BODIPY derivatives are necessary for future *in vivo* applications.

Sozmen et al. developed a library of styryl-conjugated BODIPY probes (EUA1-5, Figure 1.10) with emission wavelengths ranging from 654 to 763 nm for optical imaging of A β plaques.¹²¹ The measured K_d values of EUA1-5 were 320, 230, 320, 48.6 and 97 nM, respectively. Among them, **33** (EUA-1), **34** (EUA-2) and **35** (EUA-4) efficiently stained A β plaques in Tg2576 mice brain sections and demonstrated good BBB penetration.¹²¹ The probe, **35**, the highest affinity and best fluorescence staining, would be a promising diagnostic agent after further modification.

Figure 1.10 Chemical structures of BODIPY derivatives.¹¹⁸⁻¹²¹

Alkatratriene derivatives

Recently, Ono, Cui and co-workers developed a new series of structurally simplified A β fluorescent probes DANIRs, with a traditional donor (*p*-dimethylamino phenyl moiety)-acceptor (dicyanomethylene) structure bridged by an alkatratriene chain.¹²²⁻¹²³ This design significantly reduced molecular weights of the probes and was reported to improve its pharmacodynamics. Among them, the best probe, **36** (DANIR 2c, Figure 1.11) showed favourable optical properties (665 nm emission wavelength), a 12-fold increase in intensity upon binding to A β aggregates, and excellent affinity for A β aggregates ($K_i = 37$ nM, $K_d = 27$ nM). Further studies indicated that it was able to efficiently penetrate the BBB and label A β plaques with a fast washout rate of the unbound probe. This probe could differentiate between transgenic and wild type mice as early as 30 min after *in vivo* administration. **36** meets most of the requirements as an optimal probe for *in vivo* imaging of A β plaques.¹²² The blue-shift of the probe emission wavelength to 625 nm (shorter than 650 nm required for good *in vivo* imaging) upon binding to A β plaques is one of the shortcomings, which limits the *in vivo* applications.

Inspired by the excellent performance of **36** (DANIR 2c), Cui et al. then designed and synthesized four analogs. **37** (MAAD-3, Figure 1.11), **38** (DMDAD-3, Figure 1.11), **39** (MCAAD-3, Figure 1.11) and **40** (DMMAD-3, Figure 1.11) by differing the donor group.¹²³ These probes showed extended emission wavelength and significantly reduced binding affinity to A β aggregates compared with **36**. Docking simulations revealed that these probes likely bind to the same binding site as IMPY, which has a thin hydrophobic groove parallel to the fibrillar axis formed by VAL 18 and PHE 20. Among them, **37** had the highest affinity ($K_d = 106$ nM), good brain kinetics including rapid initial uptake and fast egress and emission wavelength up to 654 nm when bound to A β aggregates, leading to a better NIRF probe for *in vivo* imaging than **36**.

Figure 1.11 Chemical structures of alkatriene derivatives.¹²²⁻¹²³

Thiophene-based derivatives

In 2005, Swager et al.¹²⁴ designed and synthesized the fluorescent probe, **41** (NIAD-4, Figure 1.12) based upon the push-pull architecture with a terminal donor (p-hydroxyphenyl group) moiety, acceptor (dicyanomethylene group) moiety and interconnected by a highly polarizable dithienylethenyl π -conjugated bridge. **41** displayed excellent binding affinity ($K_i = 10$ nM) for A β aggregates and an approximately 400-fold fluorescence enhancement, caused by reduced free rotation of aromatic rings in the excited state. Additionally, *in vivo* studies in transgenic mice indicated **41** efficiently crossed the BBB and clearly labelled both A β plaques and cerebrovascular amyloid angiopathies. However, the maximum emission wavelength of **41** is only 603 nm, outside the optimal range of 650-900 nm. Then the same group subsequently developed a series of **41** analogs, including **42** (NIAD-11, $\lambda_{em} = 690$ nm, Figure 1.12) and **43**

(NIAD-16, $\lambda_{em}=720$ nm, Figure 1.12).¹²⁵⁻¹²⁶ **42** showed a dramatic fluorescent increase and a 15-nm red-shift in the emission wavelength when mixing with A β aggregates. **43** intensely stained A β plaques in the AD mouse brain sections.

Peter Nilsson and co-workers rationally designed and synthesized a series of luminescent conjugated oligothiophenes and found that in contrast to large polythiophenes, oligothiophenes, especially pentathiophenes **44** (p-FTAA, Figure 1.12) are able to spectrally discriminate between NFTs and senile plaques.¹²⁷ Although the highly charged nature, **44** was able to efficiently penetrate the BBB. In addition, **44** labelled senile plaques showing a vibronic fine structure spectrum with double peaks at 520 and 545 nm, whereas NFTs displayed a broad featureless emission centred at 558, allowing for the identification of both aggregates using the same fluorophore in AD brain sections. When analyzing the probes, the authors found that **44** differed from the others in its solvatochromic behavior.¹²⁸ As such, **44** showed much stronger conformational restrictions on binding A β fibrils, as proved by the presence of the vibronic double peaks as well as the reduced Stokes shift, whereas the binding site on tau fibrils would be less conformationally restricted and more polar. In addition, the function of the terminal carboxylates would seem to be to extend the electronic conjugation in planar conformations of **44**. Its replacement with acetyl groups (**45**, p-KTAA in Figure 1.12) leads to a 3.75-fold stronger dependence on the solvent polarity and a larger difference between the emission wavelengths (570 nm for senile plaques and 595 nm for NFTs). And it should be noted that no vibronic peaks in the case of senile plaque binding were observed for this fluorophore.¹²⁸ In short, the presence of both inner two acetic acid groups as well as the terminal carboxylic acids were thought to be crucially important to allow for spectral discrimination.¹²⁷

Figure 1.12 Chemical structures of thiophene-based probes.¹²⁴⁻¹²⁸

Other probes

In 2005, Gremlich et al. designed and synthesized a collection of oxazine-derivative NIRF probes, including **46** (AOI-987, Figure 1.13) which could readily cross BBB and bind to A β plaques.¹²⁹ However, due to the low fluorescence enhancement upon binding to A β aggregates observed *in vitro*, the signal contrast between the wild-type and APP23 transgenic mice was not significant.

Chang et al. designed and synthesized a new family of fluorescent probes with a nitrogen donor group and a cyano-acrylate electron acceptor containing water solubilizing groups (WSGs) connected via a naphthalene unit.⁹¹ Among them, **47** (ANCA-11, Figure 1.13) showed the highest binding affinity ($K_d = 1.4 \mu\text{M}$) and a 7.7-fold fluorescence intensity increase upon binding to A β aggregates. Further *in vitro* assays demonstrated that **47** could fluorescently stain amyloid deposits in human AD brain tissues. It is found that structural modification using WSG group does not significantly affect the binding affinity.

Li et al. reported a series of styryl probes. One of these compounds, **48** (STB-8, Figure 1.13) exhibited a binding affinity of $K_d = 3.2 \mu\text{M}$ to A β fibrils *in vitro* and strong A β plaques staining in AD brain sections. In *in vitro* assays, **48** stained the A β plaques, co-stained by ThS and *in vivo* staining to overlap with Congo Red in AD transgenic mice. The results of *in vivo* and *in vitro* imaging clearly indicated its BBB permeability and specific A β plaques staining in AD mice.¹³⁰

Figure 1.13 Chemical structures of other probes.^{91, 129-130}

1.6.3. Tau fibril fluorescent probes

To date, several studies by Braak *et al.* have apparently demonstrated a much more rigorous correlation between the NFT burden and AD progression.¹³¹ Therefore, tau NFTs have become an attractive target in the development of clinical diagnostic probes, mainly based on the PET imaging.¹³²⁻¹³³ Although there is some overlap between tau probes for PET and fluorescence imaging, we will focus on the fluorescence imaging in the current section.

Thioflavin-based tau fibril ligands

In the past decade, the non-specific thioflavin probes have been the starting point for a number of probes with increased selectivity and affinity for tau NFTs in the presence of A β plaques. In this section, we will focus on the fluorescent probes whose development represent a major step forward in gaining tau selectivity.

Maruyama *et al.* proposed the hypothesis that specific-tau probes should consist of a narrow π -conjugated D–A molecule and also the length of the conjugation should ideally be larger than 13 Å. Therefore, they reported a series of π -extended thioflavin analogues: PBB1–5.¹³⁴ Some interesting aspects were observed that a) all probes exhibited a propensity to fluorescently stain tau aggregates; b) the affinity for A β aggregates decreased with decreased probe lipophilicity, and reciprocally therewith, the selectivity for tau aggregates increased. **49** (PBB-5, Figure 1.14) as the most hydrophilic and red-shifted fluorophore, showed the largest selectivity for tau aggregates with NIR fluorescence emission at 685 nm. *In vivo* assays indicated that this probe was demonstrated to enable discrimination between a transgenic human P301S mutant tau mouse model and a normal control. However, the presence of the benzothiazolium group re-

sulted in metabolic instability and a reduced BBB penetrability as compared with the other PBB probes. Another analog. **50** (PBB-3, Figure 1.14) could be as the most promising candidate for *in vivo* tau imaging with an emission peak in the 500–550 nm range. Interestingly, following intravenous injection of PBB-3, labelling of tau tangles and the diffusion through the blood vessels as well as subsequent infiltration in the surrounding tissues could clearly be seen.¹³⁴

Gu et al. designed and synthesized a collection of 2-styrylindolium based fluorescent probes including compound **51** and **52** (7a and 7b, Figure 1.14).¹³⁵ *In vitro* assays revealed that they could clearly stain tau NFTs in AD brain sections while only demonstrating faint fluorescence in the presence of A β plaques. These compounds displayed a relatively low toxicity of in a zebrafish assay, however the indolium cation would be expected to hinder efficient BBB penetration.

Kudo et al. disclosed a family of D–A type probe showing a multichromic response to either A β or tau aggregates. The first member of this family was compound **53** (BF-126, Figure 1.14), showing an emission maximum at 490 nm when bound to A β plaques and an emission maximum at 540 nm when bound to tau NFTs.¹³⁶ The π -extended analogue compound **54** (BF-188, Figure 1.14) resulted in an even larger difference with maxima of emission at 520 nm and 600 nm for A β plaques and tau NFTs, respectively.¹³⁷ However, this effect completely disappears when the benzimidazole group is replaced by the analogous benzoxazole and benzothiazole rings, suggesting the involvement of H-bond interactions in the binding modes.

The group of Peter Nilsson developed a series of oligothiophenes, especially pentathiophenes, which are able to spectrally discriminate between senile plaques and NFTs.¹²⁷ Further investigations into the chromophore led to the oligothiophene–PBB3 hybrids **55–59** (bTVBT1–5, Figure 1.14).¹³⁸ Herein, these compounds do not show dual staining for NFTs and senile plaques, but rather exhibit a very high selectivity for tau aggregates at low concentrations of dye. bTVBT1–5 showed emission maxima in the 600–630 nm range with spectral tails reaching well into the NIR window. Intriguingly, the replacement of the benzothiophene ring of

bTVBT1 by a benzimidazole resulted in high affinity senile plaque binding. The styrene bond was also determined to be significantly important, as a result of replacement with a thiophene ring either removed the probes' affinities for both NFTs and senile plaques or made the probe non-selective.

Figure 1.14 Chemical structures of thioflavin-based probes.¹³⁴⁻¹³⁸

Curcumin-based tau fibril ligands

Schmidt et al. developed a library of curcumin-like donor-acceptor-donor (D-A-D) compounds with a pyrazine, pyrimidine or pyridazine core structure. Among them, two of the pyrimidine containing compounds **60** and **61** (Figure 1.15), showed a significantly higher affinity for tau tangles over A β , 13.5- and 26-fold for **60** and **61**, respectively, in a competition assay with Thiazine Red R.¹³⁹ In *in vitro* experiments, **60** demonstrated efficient two-photon imaging. In *in vitro* tissue slices, however, **60** did reveal efficient staining of A β plaques, while not showing any fluorescence derived from NFTs. **61** failed to show any fluorescence.

Chong et al. designed and synthesized a family of curcumin-analogue compounds.¹⁴⁰ Among them, **62** (Figure 1.15) exhibited a fluorescence turn-on of 22.9-fold and was able to stain aggregates of tau 2N4R-GFP (green fluorescent protein) conjugate *in vitro*. Due to the electron-donating dimethylaniline groups and the significantly increased D–A–D intramolecular charge transfer (ICT) efficiency, the emission wavelength of **62** was red-shifted up to 620 nm. Neither *in vivo* or tissue staining experiments, nor competition experiments with A β were performed. Using the same donor moiety, the same group reported a collection of D– π –A probes **63** and **64** (Figure 1.15), displaying an impressive 49- and 108-fold fluorescence intensity increase upon binding tau aggregates.¹⁴¹ Furthermore, **63** and **64** showed a fluorescence intensity selectivity of 5.7- and 3.8-fold fold higher for tau aggregates than A β , whereas for both compounds the binding affinity is higher for A β than for tau fibrils. The probes showed a clear co-localization with GFP-labelled tau aggregates *in vitro*, staining of human brain sections did reveal the absence of fluorescence in normal brains, whereas bright fluorescence was observed in the case of AD pathology. As a result of the strong D– π –A strength, the fluorescence emission wavelength approaches the NIR window cut-off (> 650 nm).

Inspired by a strategy that has previously been proven to be successful in the CRANAD series A β -specific fluorophores, the same group synthesized a more lipophilic and brighter fluorescent probe **65** (Figure 1.15) by aromatizing the β -diketonate of the curcumin structure with difluoroboron. The D–A curcumin-like compound **65** showed a high turn-on ratio in the presence of tau fibrils with a 14-fold selectivity for tau over A β and an emission maximum in the NIR up to 660 nm. In *in vitro* assays, the molecule showed an excellent overlap with a fluorescently labelled antibody for tau tangles in human AD brain sections, whilst only displaying a relatively low staining of core A β plaques of the most dense plaques.¹⁴²

Figure 1.15 Chemical structures of curcumin-based fluorophores.¹³⁹⁻¹⁴²

BODIPY-based tau fibril ligands

Vedamalai et al. recently reported the first BODIPY-based probe **66** (Figure 1.16) with a high affinity for tau aggregates.¹⁴³ **66** displayed a 7-fold fluorescence enhancement at 579 nm upon binding to tau aggregates. No data in the presence of A β fibrils were reported.

Lim et al. reported probe **67** (BD-tau, Figure 1.16) which showed a 5.5-fold fluorescence enhancement upon the addition of tau protein aggregates *in vitro* with an emission maximum around 590 nm.¹⁴⁴ However, in the presence of A β or insulin fibrils a 3.5- to 4-fold fluorescence enhancement was observed as well. Additionally, BD-tau did reveal a clear co-localisation in tau protein expressing cells and brain sections of a tauopathy mouse model.

Verwilst et al. developed the rational design of **68** and **69** (Tau 1 and Tau 2, Figure 1.16) through the π -elongation of a known A β -selective BODIPY probe.¹⁴⁵ Solution studies revealed a fluorescence enhancement based on the surrounding solvent polarity, rather than the viscosity. It is noted that **68** and **69** showed a fluorescence enhancement of 6.4- and 9.3-fold, respectively, in the presence of tau aggregates, whereas virtually no enhancement was observed in the case of A β -fibrils. In *in vitro* experiments, **68**, exhibiting the highest solubility, indicated the efficient labelling of NFTs in both acute and transgenic mouse models for tauopathy.

and AD, with minimal labelling of the core A β plaques. In addition, the authors revealed the BBB crossing of **68** and the ability for *in vivo* staining of tau aggregation status in a transgenic mouse model compared to a control.

Figure 1.16 Chemical structures of BODIPY probes.¹⁴³⁻¹⁴⁵

Other scaffolds for tau fibril ligands

This section describes a few compounds that have no or very small structural overlap with the classes of probes described above. As the first example, compound **70** (BSc3504, Figure 1.17) showed strong interactions with tau tangles in AD brain sections, as proved by fluorescence in the DAPI window (415–475 nm), while displaying considerably less pronounced staining of senile plaques.¹⁴⁶ Interestingly, in *in vitro* experiments, the compound also exhibited a very strong tau aggregation inhibition, making this compound a potential fluorescent theranostic agent, although much longer wavelengths would be required for *in vivo* applications.

Cyanine probes have long been used in biological imaging, due to their generally high brightness with emission maxima at around 570 nm, and relatively low toxicity. Schmidt et al. developed a small library of trimethine cyanines of 2,3,3-trimethyl indolinium as more sensitive and benign probes, such as compound **71** (Figure 1.17). All compounds could intensely stain NFTs in AD brain tissues, but did not stain senile plaques in the same tissues. The reason for this observation however is not fully understood.¹⁴⁷ One potential explanation could be that the human brain contains all 6 isoforms as well as many posttranslational modifications in

NFTs, whereas tau aggregation assays usually only use one isoform of tau protein (often 2N4R).

Rejc et al. recently published a small collection of long-range D–A probes, for example compound **72** (Figure 1.17), showing good affinity to tau tangles. Whereas the compounds all displayed some degree of binding to A β , **72** displayed the lowest affinity to A β in a competition assay.¹⁴⁸ The library of compounds was pre-screened in a PHF6 docking assay, demonstrating a tight fit for all compounds in the tyrosine channel. **72** showed an emission maximum at 580 and 595 nm for senile plaques and NFTs, respectively, allowing for a visual identity of the aggregates in tissue slices (green versus orange, respectively).

Figure 1.17 Chemical structures of other probes.¹⁴⁶⁻¹⁴⁸

2. Research aim

The overall aim in the study was to synthesize and evaluate novel fluorescent stilbene-based probes for detection and imaging of disease-associated protein aggregates.

More specific aims were:

- a) To elucidate the connection between probe design and its properties as an amyloid ligand.
- b) Vary the design of amyloid ligand to change the photophysical properties of the probe.

3. Methodology

Fluorescence, a form of luminescence, is a physical phenomenon in which a particular molecule emits light of a particular color very shortly after having absorbed light. In most cases, fluorescence has a longer wavelength than that of the absorbed radiation. The Jablonski diagram in Figure 3.1 is often used to visualize this process. Fluorescence has some general rules including Kasha's rule, mirror image rule and Stokes shift, which are useful for understanding fluorescence.¹⁴⁹

Figure 3.1 Jablonski diagram of absorption and fluorescence. The blue arrows represent absorption, the dashed arrow represents internal conversion from excited state S_2 to S_1 , the short-dashed arrows vibrational relaxation, and the green arrows represent fluorescence. Both the absorption and internal conversion processes are generally very fast, within a few femtoseconds to some 10ths of picoseconds (ps), whereas fluorescence, if present, is in the order of 100 ps to 200 nanoseconds for molecules in solutions.

- 1) Kasha's rule, named after American spectroscopist Michael Kasha, stating that photon emission (fluorescence or phosphorescence) occurs in appreciable yield only from the lowest excited state of a given multiplicity.¹⁴⁹
- 2) Mirror image rule, usually the emission spectrum is a mirror image of the absorption spectrum.¹⁴⁹

- 3) Stokes shift, the emitted fluorescence light has a longer wavelength than the absorbed light. This phenomenon is due to energy loss, usually to vibrations and electrostatic relaxation of the environment.¹⁴⁹

3.1. Fluorescence spectroscopy

Fluorescence spectroscopy (also known as fluorometry or spectrofluorometry) is a type of optical spectroscopy that analyzes emitted light including emission wavelengths and the strength of different wavelengths.¹⁴⁹ It involves using a beam of light, usually ultraviolet to visible light, that excites the electrons in molecules of certain compounds and causes them to emit light of longer wavelength. The background signal is generally low as the emission is collected at other frequencies than that of the excitation.

The emission wavelength maximum (λ_{em}) is a main feature of the fluorescence spectrum. It's important to note that λ_{em} is sensitive to the environment, usually the organic solvent. If the solvent is polar, then more energy is required to reorient their dipoles, and if the solvent is apolar, then the excited state will induce the dipole formation in the solvent, which requires less energy. After the relaxation the excited molecule will then decay by emitting a photon, which will thus have a longer wavelength than the absorbed photon, and this difference is called the Stokes shift. General speaking, the more polar the environment is for a given fluorophore, the longer stokes shift becomes. For similar reasons, the lifetime of the fluorescence is usually shorter in the polar environment than in the nonpolar environment, although there are exceptions to these rules. The well-known text-book of Lackowicz 'Fluorescence Spectroscopy', (Springer Verlag) gives a comprehensive introduction to the topic including many examples applied in biophysically related sciences.

3.2. Fluorescence microscopy

The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, such as an epifluorescence microscope with a more simple set up or a confocal microscope with a more complicated design. In brief, instead of using a bright white light source going through the sample through the condenser under the sample holder, the excitation light is normally coming from above onto the sample. The emitted light is collected as normal, but the scattered excitation light is filtered away by using dichroic filters (see Figure 3.2). Due to the importance of fluorescence microscopy for chemistry, Eric Betzig, William Moerner and Stefan Hell were awarded the Nobel Prize in chemistry for "the development of super-resolved fluorescence microscopy" in 2014.

Figure 3.2 The principle of LSM 780 confocal and multiphoton laser scanning microscopy (*picture from ZEISS*).

In this thesis, we use this technique to visualize the location of a certain protein in a tissue when labelled by fluorescent probes. The most frequently used imaging technique is confocal fluorescence microscopy. In a confocal microscope, excitation of your sample is performed point-by-point using a scanner, together with the use of a pinhole, which eliminates

out-of-focus signals. This gives you a much better optical resolution compared to conventional fluorescence microscopy, where the whole sample is flooded with light at once.¹⁵⁰ Tissue samples used throughout this thesis were produced according to the way that the tissue is embedded in Tissue-Tek O.C.T. and cut in thin sections (10-20 μm) using cryo-sectioning. When preparing the tissue for histology analyses one usually starts by fixing the sample to ensure that the tissue is stabilized and maintains its cellular structure. Thereafter the sample is stained, washed to remove the unbound probe and mounted on special slides for microscopy.

3.3. Time-correlated single-photon counting (TCSPC)

Fluorescence lifetimes were determined using the time-correlated single photon counting (TCSPC) technique. Here a high pulse repetition laser diode (or lamp) is used as excitation source. The system measures the time between the excitation pulse is sent out, until the emitted light reached the detector. By repeating this many times a histogram representing the life-time decay is obtained. As seen in Figure 3.3, the TCSPC technique is based on the principle that the statistical probability distribution for the emission of a single fluorescent photon is equivalent to the actual intensity versus time distribution for all photons emitted, the so called ergodic principle.¹⁴⁹

Figure 3.3 Principle and scheme of the TCSPC experiment.

Fluorescence decays were measured using an IBH-TCSPC spectrometer. Even though short pulsed excitation ($\leq$ ns duration) is used, and the system has short response time in the TCSPC mode, the relaxation times are of comparable orders of magnitude. This requires an additional recording of the instrumental response function (prompt) of the excitation pulse, prior to any analysis of the decay curve, so called deconvolution fitting, using the commercial spectrometer software (IBH). The decays were assumed to be a sum of exponentials (eq. 1).

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

Here α_i are the pre-exponential factors associated with the decay time τ_i . The quality of the fit was evaluated from the χ^2 parameter and the random distribution of the residuals as well as the autocorrelation of the residuals. The average lifetime of fluorescence decay was calculated according to the following equation,

$$\tau_{avg} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (2)$$

3.4. Determination of fluorescence quantum yield

In this work, fluorescence quantum yield (QY) was calculated using a relative determination based on a standard sample with known QY.¹⁵¹ QY is then determined using the following equation:

$$\Phi_{\text{sample}} = \Phi_{\text{stand}} (F_{\text{sample}} / F_{\text{stand}}) \times (A_{\text{stand}} / A_{\text{sample}}) \times (n_{\text{sample}}^2 / n_{\text{stand}}^2)$$

Where Φ = Quantum yield, F = Integrated area of the fluorescence spectrum, A = Absorption maximum, and n = Refractive index of solvent. Quinine hemisulfate salt monohydrate ($\Phi = 0.58$ in 0.1M H_2SO_4), fluorescein ($\Phi = 0.95$ in 0.1M NaOH) and coumarin 314 ($\Phi = 0.68$ in ethanol) were used as the standards in this work. The quantum yields of these standards are mostly independent of the excitation wavelength, allowing them to be used wherever they display useful absorption. We chose the best standard for each ligand according to its absorption.

3.5. Dissociation constant (K_d) by fluorescence spectroscopy

In this thesis the binding affinity of ligands to fibrils or fibrils to ligands (due to the low solubility in water, paper I) was investigated by measuring the dissociation constant (K_d) using fluorescence spectroscopy. The K_d -value from fluorescence spectroscopy was determined by adding ligands to fibrils or fibrils to ligands and the fluorescence signal was expected to change upon binding. The change in emission intensity was then plotted versus ligand concentration (paper II-IV) or fibril concentration (paper I).

The following equation describes the binding of a ligand to a protein:

The dissociation constant K_d is defined at equilibrium by

$$K_d = \frac{[P][L]}{[PL]}$$

Where $[P]$ is concentration of free protein, $[L]$ is concentration of free ligand and $[PL]$ is the concentration of the PL complex.

The K_d binding curve was generated by GraphPad Prism 7.0 by using the following equations:

$$Y = B_{\max} * X / (K_d + X)$$

$$Y = B_{\max}Hi * X / (K_dHi + X) + B_{\max}Lo * X / (K_dLo + X)$$

Where X is concentration of ligand and Y is change in fluorescence intensity. $B_{\max}$ is the maximum specific binding having the same unit as Y .

4. Summary of papers

4.1. Paper I: *Trans*-stilbenoids with extended fluorescence lifetimes for the characterization of amyloid fibrils

Aim: The aim of the first paper was to synthesize two new *trans*-stilbenoids with expanded aromatic ring systems (anthracene and pyrene). The series of four analogous *trans*-stilbenoids with benzene, naphthalene, anthracene, and pyrene were investigated with respect to their photophysical properties free in solution and when bound to amyloid fibrils (insulin, lysozyme and A β 1-42), including time-resolved fluorescence measurements.

Results and discussion: Previously, we successfully reported that two naphthyl-based *trans*-stilbene probes, **73** and **75**, can bind both native TTR and misfolded proto-fibrillar TTR at physiological concentrations, displaying distinct shift in emission maxima (>100 nm), allowing conformational discrimination by fluorescence spectroscopy.¹⁵² Herein, this class of compounds having a *trans*-stilbene or *trans*-stilbenoid structure are indeed a promising fluorescent probe scaffold for amyloid research. Accordingly, we designed and prepared two new analogues (**76** and **77**, see the structures in Figure 4.1) with expanded aromatic ring systems (anthracene and pyrene), to investigate their spectral discrimination of binding native and proto-fibrillar TTR as well as three other canonical amyloid proteins (insulin, lysozyme and A β 1-42). Subsequently, the emission properties of these analogues (**74–77**) with different aromatic ring moieties (benzene, naphthalene and anthracene as well as pyrene) were investigated using steady-state and time-resolved fluorescence spectroscopies, which demonstrate the feasibility of these new scaffolds for amyloid fibril characterization.

Figure 4.1 Chemical Structures of *trans*-Stilbenoids: **73**, **74**, and **75** have been reported previously by our group.¹⁵² **76** and **77** were synthesized in this paper.

Synthesis procedures: Pyrene derivatives are important and well-studied organic chromophore with long excited state lifetime. Structural modifications allow for many applications, including fluorescent probes,¹⁵³ biological labeling,¹⁵⁴ molecular electronics,¹¹¹ photovoltaic cells, and field-effect transistors.¹⁵⁵ Most reported pyrene derivatives involve substitution at the 1,3,6,8-positions and/or functionalization at the 4,5,9,10-positions. To substitute pyrene at the 2- or 7-positions is synthetically challenging because of the presence of the nodal plane of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), which lies perpendicular to the molecular plane and passes through the 2- and 7-positions.¹⁵⁶ However, pyrene derivatives with substituents at the 2,7-positions are particularly interesting. These derivatives have a geometrically linear molecular structure that is useful for the construction of large optoelectronic systems with well-defined architectures.

The synthesis of 2,7-substituted pyrene derivatives often requires tedious multistep procedures.¹⁵⁷ Recently, Marder et al. have reported the synthesis of 2- and 2,7- functionalized pyrene derivatives including compounds **79** and **80**.¹⁵⁸ Our approach for the synthesis of the *trans*-stilbene derivatives **76** and **77** was to apply the palladium-catalyzed Heck reaction of **82** to the corresponding **80** (2-bromopyrene) and **83** (2-bromoanthracene). Employing the catalyst that was prepared *in situ* by the reaction of [Ir(u-OMe) cod]₂ with 4,4'-di-*tert*-butyl- 2,2'-dipyridyl (dtbpy), the product **79** was obtained in 62% yield. Then, the reaction of compound **79** with 3 equiv of CuBr₂ in methanol/H₂O (1:1) gave **80** in 78% yield. From **81** (3,4-dihydroxybenzaldehyde), **82** was obtained by a witting reaction

in 59% isolated yield, in which the Heck reaction with **83** and **80** yielded the target molecules **76** and **77** in 35% and 28% isolated yields, respectively. Figure 4.2B depicts the synthesis of the target molecules.

Figure 4.2 A) Position numbering and HOMO and LUMO plots of pyrene. B) Synthesis of compounds **76** and **77**.

Both probes, **76** and **77**, can bind to both native and proto-fibrillar TTR, displaying indiscriminate shift in emission maxima when bound to the two different TTR conformations. The amyloid-binding propensity for four analogous *trans*-stilbenoids (**74-77**) with different aromatic ring moieties was compared. All studied probes can bind specifically to amyloid fibrils of vastly heterologous proteins (insulin, lysozyme, TTR, and A β 1-42 fibrils) with apparent fluorescence intensity augmentation and moderate affinities. One specific aim was to develop long lifetime fluorescent probes to allow measurements of solution dynamics of amyloid species. To our knowledge, no small-molecule amyloid targeting probes have been reported with pyrene or anthracene directly binding to the amyloid fibril binding sites. The fluorescence lifetimes of the amyloid

probes described in this work in degassed methanol are ranked as (**74** < 0.05 ns) < **75** = 0.52 ns < **76** = 4.23 ns < **77** = 19.11 ns, indicating that the different aromatic ring moieties determine the respective probe residence time in the excited state. When bound to A β 1-42 fibrils, the lifetimes are 1.3 ns (**74**), 1.5 ns (**75**), 5.7 ns (**76**), and 29.8 ns (**77**). Hence, each probe bound to A β 1-42 fibrils showed longer lifetime than in degassed methanol, suggesting that the probe binding sites are hydrophobic and well-shielded from the surrounding water solvent. The pyrene-based **77** showed the longest fluorescence lifetime, opening the opportunity for size determination of the oligomerization process by dynamic fluorescence spectroscopy.

Figure 4.3 Emission spectra of (A) **74**, (B) **75**, (C) **76**, and (D) **77** bound to either native insulin or insulin fibrils in PBS buffer (pH 7.4). 1 μ M was used for probe and 5 μ M (on a monomer basis) was used for native insulin and insulin fibrils. Ex = 330 nm for all probes. *Reproduced with permission from ACS Omega 2017, 2, 4693-4704.*

In summary: To make a palpable overall comparison of the probes against various amyloid fibrils, we generated a starplot (Figure 4.4A) to compare various K_d values and fluorescence increase for the respective probe. This graphical representation illustrates that **76** shows the strongest fluorescence increase for fibrils at acidic pH and that the affinity (low K_d) generally increases as the hydrophobicity of the probe increases, with the strongest binding for **77**. Furthermore, a star-plot representation of the photophysical properties including Stokes shift and fluorescence lifetimes clearly display a similar separation with highest Stokes shift for **76** and the longest fluorescence lifetimes for **77** (Figure 4.4B). We foresee that this class of probes will be useful for amyloid fibril research.

Figure 4.4 Star-plot representation to compare all probe parameters in graphical format. (A) Binding properties including dissociation constants (K_d in μM) of the probes against different fibril types and the fold increase of fluorescence intensity of the fibril-bound probe compared with the free probe. (B) Star-plot representation of the photophysical properties including Stokes shift (nm) and average fluorescence lifetime (τ_{avg} in ns) of the probes. *Reproduced with permission from ACS Omega 2017, 2, 4693-4704.*

4.2. Paper II: Intramolecular proton and charge transfer of pyrene-based *trans*-stilbene salicylic acids applied to detection of aggregated proteins

Aim: The aim of the second paper was to synthesize two pyrene-based *trans*-stilbene salicylic acids (Py1SA and Py2SA). The emission spectra of both molecules when bound to native and fibrillar A β 1-42 were investigated and their intrinsic photophysical properties were also studied in different solvents [DMSO, ethanol (EtOH) and toluene] for a wide range of concentrations by steady-state absorption and emission.

Results and discussion: Our approach for the synthesis of the *trans*-1 and 2-styryl-pyrene derivatives (Py1 and Py2 derivatives) was to apply the palladium-catalyzed Heck reaction of styrene derivatives to the corresponding **84** (1-bromopyrene) and **80** (2-bromopyrene). The first synthesis of **84** was reported in 1937 by Lock, via the bromination of pyrene parent.¹⁵⁹ Then similar approaches have been developed using reagents such as NBS,¹⁶⁰ CuBr₂,¹⁶¹ and HBr with H₂O₂.¹⁶² In this work, **84** was

Scheme 4.1 Synthesis of *trans*-1 or 2-styryl-pyrene derivatives (Py1 and Py2 derivatives). Reagents and conditions: a) HBr (48%), H₂O₂ (30%), MeOH/Et₂O (1:1), rt, 92%; b) B₂Pin₂ (1.1 equiv), Ir (u-OMe) cod₂ (1% mol), dtbpy (2% mol), hexane, 80 °C, overnight, 60%; c) CuBr₂ (3 equiv.), MeOH/H₂O (1:1), 90 °C, overnight, 75%; d) Ph₃PCH₃Br (1.5 equiv.), tBuOK (4 equiv.), THF, rt, 6 h; e) Pd(OAc)₂ (10% mol), triethanolamine (2 equiv.), DMF, 100 °C, 24 h. [Ir(μ-OMe)cod]₂ = (1,5-Cyclooctadiene)(methoxy)iridium (I) Dimer, dtbpy = 4,4'-Di-tert-butyl-2,2'-dipyridyl.

synthesized in 92% yield under the conditions: HBr (48%), H₂O₂ (30%), MeOH/Et₂O (1:1), room temperature. And **80** was obtained using the same approach.¹⁶³ Finally, *trans*-1-styryl-pyrene derivatives: **90** (Py1Bz), **91** (Py1BzOH) and **92** [Py1Bz(OH)₂] as well as **93** (Py1SA) were prepared in 57%, 66%, 53% and 70% yields, respectively. *Trans*-2-styryl-pyrene derivatives: **94** (Py2Bz) and **95** (Py2SA) were obtained in 35% and 43% yields.

We first investigated the utilities of both Py1SA and Py2SA as probes for detecting A β 1-42 aggregates in PBS. As can be seen from the fluorescence spectra shown in Figure 4.5 a bright fluorescence is switched on upon mixed with A β 1-42 fibrils, but with entirely different emission bands, as reflected using a fluorescence images with distinct colors. Py1SA showed a broad emission around 498 nm while Py2SA displayed the characteristic pyrene emission spectrum with resolved vibrational substructure having the maximum at 419 nm. To assess if this was specific for A β 1-42 fibrils we also assayed four other amyloid fibrils (insulin, lysozyme and Prion as well as tau) and observed very similar emission patterns even at low pH.

Figure 4.5 Emission spectra of Py1SA (A) and Py2SA (B) upon bound to native, fibrillar and oligomeric forms of A β 1-42 in PBS buffer (pH 7.4). 5 μ M (on a monomer basis), was used for native, fibrillar and oligomeric forms of A β 1-42. 1 μ M was used for probes. λ_{ex} = 390 nm (Py1SA), 330 nm (Py2SA). Insets: fluorescence images of mature A β 1-42 fibrils stained by probes.

Then we investigated the geometric, LUMO structures and photophysical properties of Py1SA and Py2SA in three organic solvents considered

(DMSO, EtOH and toluene). As shown in Figure 4.6, the phenyl and pyrene groups of Py1SA are twisted along the ethylene group, whereas

Figure 4.6 Geometric (A), LUMO structures (B) and absorption in DMSO (C) as well as emission spectra in DMSO (D) of Py1SA and Py2SA.

Py2SA is nearly planar. The structural difference between Py1SA and Py2SA is consistent with the previous observation that the 2-position of pyrene is more sterically accessible to substitution than the 1-position.¹⁶⁴⁻¹⁶⁵ The LUMO of Py1SA indicates that the molecular twist does not significantly disturb the conjugation of Py1SA, which is in line with previous results^{111, 166-167}, whereas the LUMO of Py2SA is more localized on the pyrene moiety with only a minor contribution from the SA carbon at the pyrene 2-position, suggesting that in Py2SA, the electronic coupling between the pyrene and SA moieties is weaker.^{165, 167} Consistent with the strong influence on both the S₂←S₀ and S₁←S₀ excitations observed for the 1-substituted pyrene derivatives,¹⁵⁶ the absorption spectrum of Py1SA in DMSO showed a broad band (395 nm) with no clearly defined S₂←S₀ and S₁←S₀ excitations (Figure 4.6C). While the absorption spectrum of Py2SA exhibited a pyrene-like S₂←S₀ excitation (343 nm) with a small

bathochromic shift (3 nm) from the $S_2 \leftarrow S_0$ transition in pyrene (Figure 4.6C). Furthermore, the emission spectra of 5 μM Py1SA and Py2SA in DMSO are shown in Figure 4.6D. Py1SA only showed a broad, featureless emission spectrum centered at 555 nm, called "K emission", which is assigned to the ESIPT and ICT state of anionic K-form (anionic K_{ESIPT} -form + anionic K_{ICT} -form, Scheme 4.2) according to previous reports.¹⁶⁸ While Py2SA showed two apparent emission bands: one around 565 nm, namely, the K emission, the other around 416 nm regarded as "E emission", clearly to be attributed mainly to the locally excited (LE) state of the pyrene moiety of the neutral E-form.

Scheme 4.2. A) The ground- and excited-state species of Py1SA and Py2SA as well as a schematic of their photo-physics. B) Different species of Py1SA and Py2SA in ground state. *Reproduced with permission from Chemphyschem, 2018, 19, 1-10.*

In summary: The DFT calculations showed that Py1SA is twisted while Py2SA is more planar. The LUMOs indicated that Py1SA has a large coefficient on the connecting carbon at the 1-position, whereas Py2SA has a low coefficient on the connecting carbon at the 2-position, reflecting that the pyrene is connected covalently but uncoupled electronically. Spectroscopic experiments showed that Py1SA and Py2SA at low concentrations, on photo-excitation exhibit dual emission corresponding to normal (E-emission) and large Stokes-shifted fluorescence (K-emission) in polar solvents. The former is attributed to a locally excited state of neutral E-form, while the latter includes ESIPT and ICT emission. We conclude that K-emission is observed only in the anionic E-form in a polar solvent. We demonstrated that both compounds can discriminate between native and fibrillar forms of A β 1-42 and can work as probes for quantitative analysis and kinetics study of amyloid fibril formation. Py1SA showed a broad emission band around 498 nm, attributed to the strong ICT between pyrene and benzene ring, not ESIPT when bound to preformed A β 1-42 fibrils or other amyloid fibrils (insulin, lysozyme, Prp and Tau). While Py2SA displayed a pyrene-type emission spectrum with a sharp peak 419 nm due to the nodal plane through the 2- and 7-position of pyrene. The different spectral outputs arising from distinct ICT of the two ligands when bound to five different amyloid proteins shows that minor alterations in probe design can be used for tuning the fluorescence properties of amyloid probes.

4.3. Paper III: Detection and imaging of A β 1-42 and Tau fibrils by redesigned fluorescent X-34 analogues

Aim: the aim of the third paper was to synthesize four new ligands with distinct optical properties by replacing the central benzene unit of the Congo red analogue X-34, with other heterocyclic moieties (naphthalene, thiophene and quinoxaline as well as benzodithiazole) for imaging Alzheimer's disease (AD) pathology comprising A β and Tau aggregates.

Results and discussion: Our approach for the synthesis of 4 new ligands was to apply the palladium-catalyzed Heck cross-coupling reaction of compound **89** with dibrominated **96** (naphthalene), **97** (thiophene) and **98** (quinoxaline) as well as **99** (benzodithiazole) to give **100** (NSB), **101** (TSB), **102** (QSB), and **103** (BTDSB) with final yields of 38%, 64%, 58%, and 72%, respectively (Scheme 4.3). Compound **89** was synthesized according to the methods described.¹⁶³

Scheme 4.3 Synthesis of NSB, TSB, QSB, and BTDSB. Reagents and conditions: a) Pd(OAc)₂ (10% mol), triethanolamine (4 equiv), dry DMF, 100 °C, 24 h. *Re-produced with permission from Chem. Eur. J., 2018, 24, 7210-7216.*

Then *in vitro* ligand binding assays with aggregated A β 1–42 or tau were investigated as demonstrated in Figure 4.7A-C. X-34 was included for comparison in this study. All ligands exhibited significant redshifts in absorption spectra of 20, 15, 10, 12, and 17 nm, respectively, upon binding

to A β 1-42 fibrils, in agreement with that of Congo red mixed with A β 1-40 or 1–42 fibrillar aggregates.¹⁶⁹ This shift is assigned to fibril-induced molecular planarization of the ligand and subsequent increase in hyper-conjugation. These ligands showed different degrees of fluorescence changes in the presence of A β 1-42 or tau fibrils. Especially, QSB and BTDSB showed small fluorescence changes when mixed with tau fibrils. The quantum yields of ligands bound to A β 1-42 fibrils significantly increased compared to those of free ligands in PBS, suggesting that fibril

Figure 4.7 Absorption (dashed line) and emission (solid line) spectra of X-34 (A), TSB (B) and BTDSB (C) in the absence/presence of A β 1-42 peptide (monomer, oligomer and fibril) or tau fibrils in PBS buffer (pH 7.4). 5 μ M fibrils and 500 nM probe were used for all experiments. Absorption spectra are normalized by the peak value. Emission spectra are presented as relative intensities. (D) Quantum yield (QY) of ligand (ThT, X-34, NSB, TSB, QSB and BTDSB) in the absence/presence of A β 1-42 fibrils in PBS buffer (pH 7.4). Free probe in degassed MeOH is also shown. Values represent the average of three determinations. The same legend as in (A) is used in (B) and (C). *Reproduced with permission from Chem. Eur. J., 2018, 24, 7210-7216.*

bound ligands were preorganized in a geometry which is close to the planarized geometry of the emissive excited state after it has reached its equilibrium (Figure 4.7D).

We then measured the binding affinity of the ligands to A β 1-42 or tau fibrillar aggregates and the competition with radiolabeled ligands, ^3H -PiB and ^3H -X-34, which bind to distinct sites on A β fibrils.¹⁷⁰ The detailed binding constants of ligands with aggregated A β 1-42 or tau are summarized in Table 4.1. From the binding competition, the ligands did not compete with ^3H -PiB but it was evident that the nature of the central chemical moiety had a major influence on the X-34 derivatives efficiency to displace ^3H -X-34 from recombinant A β 1-42 fibrils. In comparison with X-34, introducing a thiophene group (a weak electron-donating group) decreased the EC₅₀ value, whereas introduction of quinoxaline (Q) and benzodithiazole (BTD) (two electron-withdrawing groups, Q < BTD), successively increased the EC₅₀ values. Additionally, for Tau fibrils all ligands predominantly competed with ^3H -X-34 with lower EC₅₀ values compared to that for A β 1-42 fibrils.

Table 4.1 Binding constants of ligands with aggregated A β 1-42 or tau

Ligand	K _d (nM) ^[a]		EC ₅₀ (nM) ^[b]	
	A β 1-42	Tau	A β 1-42	tau
ThT	665 $\pm$ 66	2873 $\pm$ 144	N.D.	5000
X-34	299 $\pm$ 21	195 $\pm$ 10	80	10
NSB	264 $\pm$ 13	175 $\pm$ 7	750	22
TSB	13 $\pm$ 1	16 $\pm$ 1	16	12
QSB	124 $\pm$ 7	155 $\pm$ 6	120	60
BTD SB	93 $\pm$ 5	110 $\pm$ 5	140	12

^[a]K_d values were determined by saturation binding assays by fluorescence. Values represent the average $\pm$ SD of two determinations. ^[b]EC₅₀ values of ligands competition for radioligands ^3H -X-34 binding to A β 1-42 or tau fibrils. N.D. = Not determined. *Reproduced with permission from Chem. Eur. J., 2018, 24, 7210-7216.*

Next, the X-34 derivatives, including X-34, were evaluated as fluorescent ligands in human brain tissue sections with AD pathology (Figure 4.8). All ligands exhibited specific binding to extracellular A β plaques in the brain parenchyma and intracellular NFTs, except for QSB, which selectively and specifically only stained A β plaques.

Figure 4.8 Fluorescent derivatives of the X-34 (top structure) with distinct central chemical motifs render fluorescent ligands that can be utilized to assign A β plaques and NFTs in human AD brain sections.

In summary: We have displayed a general synthetic route, where by the central benzene unit is replaced with other heterocyclic motifs, can be used for synthesizing a selection of ligands that can be utilized for fluorescence detection of protein aggregates. We have indicated that these ligands competed for binding of ^3H -X-34, not ^3H -PiB, on pure recombinant A β 1-42 amyloid fibrils and hence retained its main binding site, being of Congo red type. The nature of the central chemical moiety appeared to be a key determinant for binding affinity and for displacing ^3H -X-34 binding from A β 1-42 fibrils. Most importantly, the specific amyloid staining property of A β plaques and NFTs in human AD brain tissue was retained. We foresee that this scaffold, while rather well studied, may continue to serve a role for design of high-affinity ligands targeting A β and tau pathology in human AD brain. Especially targeting A β pathology independently of the PiB binding site via ligands such as QSB could potentially identify different A β fibril polymorphs involved in various AD subtypes.

4.4. Paper IV: Phenolic bis-styrylbenzo[c]-1,2,5-thiadiazoles as fluorescent probes for mapping microscopic A β plaque heterogeneity

Aim: The aim of the forth paper was to design and synthesize six 2,1,3-benzothiadiazole (BTD)-based ligands with a series of bis-styryl-BTDs and different conjugated spacers to investigate their A β binding affinities (A β 1-42 and A β 1-40 fibrils) and specificity towards A β amyloid plaques.

Results and discuss: We synthesized a series of BTDSB analog. **107** to **116** via the classic Heck reaction using a new one-pot procedure as depicted in Figure 4.9A. Compounds **104** to **106** were prepared via Wittig reaction of the corresponding aldehyde and triphenylphosphine methyl bromide utilizing a previous procedure.¹⁶³ Compound **87** was purchased from Sigma-Aldrich. Compounds **88** and **82** has been reported previously by our group.¹⁷¹

Figure 4.9 A) Synthesis of bis-styryl-BTDs. B) Chemical structure of DF-9.

Next, we investigated the emission behavior and binding affinity of **107** to **116** and BTDSB towards A β 1-42 fibrils and A β 1-40 fibrils, using DF-9 (see the structure in Figure 4.9B) and X-34 as references (Table 4.2). We found that these ligands showed larger fluorescence intensities when bound to A β 1-42 fibrils than bound to A β 1-40 fibrils. It is noticeable that these ligands showed higher binding affinity towards recombinant A β 1-42 fibrils compared to A β 1-40 fibrils, which is consistent with their higher fluorescence enhancement for A β 1-42 fibrils. Therefore, we will focus on the K_d values towards A β 1-42 fibrils. We note that **109** had the strongest binding affinity (19.7 ± 1.6) of all ligands. The binding affinity for **107** and **108** was 3.4-fold and 2-fold less compared to **109**, most likely due to the loss of H-bond stabilization. The K_d data for **109** versus **114** and **116** suggest that the *para*- and *ortho*- are marginally superior to *meta*-substitution on the outer phenyl rings. Although the *meta*-OH could decrease the binding affinity, **112** showed higher binding affinity compared to **114**, attributed to the 2 more of H-bond stabilization by the phenol functional group. As evidenced by the 4.3-fold loss in binding affinity for BTDSB compared to **109**, the carboxylic acid functional groups decreased the affinity, suggesting that acidic functional groups are not required for good A β binding, which is in line with X-34 (149 ± 8 nM) versus DF-9 (137 ± 10 nM) for A β 1-42 fibrils and comparable affinities for A β 1-40 fibrils.

Due to the highest binding affinities of **109** towards A β 1-42 and A β 1-40 fibrils, we then synthesized compounds **117** to **120** by means of palladium-catalyzed cross-coupling reactions of 4,7-dibromo-2,1,3-benzothiadiazole (**99**) (Scheme 4.4). **117** was achieved in 81% yield for two same steps by the Suzuki reaction as shown in Scheme 4.4. Actually, to synthesize **117** in one step using **99** and 3-fold **121** failed and only gave the mono-substituted product **122** in 89% yield. The Sonogashira coupling reaction of **99** with **123** gave compound **124** in 92% yield according to the previously reported procedures,¹⁷² then **124** was deprotected using KOH in MeOH to obtain the intermediate product, which was instantly derived to **118** by reaction with **125** (4-iodophenol) in 32% yield. The borylation of compound **126** using **127**, PdCl₂(dppf), dppf, KOAc, in 1,4-dioxane was achieved according to the previously reported patent,¹⁷³ then reacted with **99** by Suzuki coupling reaction to give the desired molecule

Table 4.2 Binding properties of BTD derivatives.

compd.	K_d (nM) ^[a]		FI ^[b]		clogP ^[c]
	A β 1-42	A β 1-40	A β 1-42	A β 1-40	
DF-9	137 $\pm$ 10	58 $\pm$ 7	16.7 $\pm$ 1.5	1.3 $\pm$ 0.4	6.2
BTDSB	83 $\pm$ 6	~250	24.3 $\pm$ 2.1	3.7 $\pm$ 0.5	8.1
107	66 $\pm$ 8	109 $\pm$ 19	2.9 $\pm$ 0.2	1.1 $\pm$ 0.1	7.3
108	39 $\pm$ 1	61 $\pm$ 17	16.1 $\pm$ 0.7	4.4 $\pm$ 0.3	6.7
109	20 $\pm$ 2	46 $\pm$ 2	17.2 $\pm$ 0.3	11.7 $\pm$ 1.3	6.0
110	39 $\pm$ 4	187 $\pm$ 8	19.7 $\pm$ 1.0	4.8 $\pm$ 0.8	4.8
111	31 $\pm$ 4	110 $\pm$ 22	21.3 $\pm$ 1.2	7.0 $\pm$ 0.9	5.4
112	95 $\pm$ 1	284 $\pm$ 12	26.7 $\pm$ 1.4	2.2 $\pm$ 0.4	4.7
113	73 $\pm$ 7	170 $\pm$ 13	48.5 $\pm$ 1.6	12.4 $\pm$ 0.6	5.3
114	116 $\pm$ 10	133 $\pm$ 10	17.2 $\pm$ 1.0	5.4 $\pm$ 0.7	6.0
115	77 $\pm$ 1	83 $\pm$ 20	9.2 $\pm$ 0.7	2.7 $\pm$ 0.2	5.4
116	29 $\pm$ 5	106 $\pm$ 22	3.6 $\pm$ 1.3	1.5 $\pm$ 0.7	6.0
117	511 $\pm$ 29	250 $\pm$ 25	7.3 $\pm$ 1.1	2.1 $\pm$ 0.7	4.4
118	36 $\pm$ 3	59 $\pm$ 5	4.6 $\pm$ 0.9	2.7 $\pm$ 0.2	5.9
119	40 $\pm$ 4	> 400	5.1 $\pm$ 1.3	2.6 $\pm$ 0.3	8.2
120	10 $\pm$ 1	> 400	3.5 $\pm$ 0.2	1.8 $\pm$ 0.5	8.4

^[a] K_d values were determined by saturation binding assays by fluorescence. Values represent the average $\pm$ SD of 2 determinations. ^[b]Fold increase (FI) = fluorescence intensity of the ligand bound to A β 1-42 or A β 1-40 fibrils / intensity of the unbound ligand in PBS. Values represent the average $\pm$ SD of 2 determinations. ^[c]Calculated cLogP values using chemdraw ultra 12.0.

119 in 21% yield. The Heck reaction of **99** and **129** produced compound **130** in 80% yield, then bromination to give compound **131** in 75% yield according to the previously reported procedures.¹⁷² And then **131** reacted with **121** by Suzuki reaction to obtain the desired molecule **120** in 46% yield.

We also investigated the emission behavior and binding affinity of **117** to **120** as demonstrated in Table 4.2. These ligands showed an apparent fluorescence intensity with significant hypsochromic shift in the presence of A β 1-42 fibrils than that when bound to A β 1-40 fibrils, demonstrating that these probes are more sensitive to A β 1-42 fibrils over than A β 1-40 fibrils. Then, we measured the binding affinity of **117** to **120** towards A β 1-42 fibrils and A β 1-40 fibrils. As shown in Table 4.2, the binding affinity of these probes, in conjuncture with **109**, towards A β 1-40 fibrils is weaker than towards A β 1-42 fibrils, which is consistent with the low fluorescence intensity enhancement upon bound to A β 1-40 fibrils. Therefore we

Scheme 4.4 Synthesis of BTDs with different spacers. Conditions and reagents: (a) Pd(PPh₃)₄, 2M Na₂CO₃, benzene-EtOH, 90 °C; (b) Pd(PPh₃)₄, CuI, triethylamine, 75 °C; (c) 1) KOH in MeOH, rt; 2) Pd(OAc)₂, PPh₃, CuI, triethylamine, THF, rt; (d) PdCl₂(dppf), dppf, KOAc, 1,4-dioxane, 90 °C; (e) NBS, CHCl₃, rt.

will focus on the binding affinity of these probes to Aβ1-42 fibrils. The symmetrical **117** had the weakest binding affinity all the ligands, 26-fold weaker than that of **109** and 3-fold less than that of DF-9. This could be attributed to the shorter distance between phenol functional groups, reducing the effectiveness of 4 to form strong H-bonds with the Aβ1-42 fibril binding pockets.¹⁷⁴ Compared to **109**, the more rigid linear alkyne analogous **118** had a 2-fold lower Aβ1-42 fibril binding affinity, possibly

due to an inability to attain a con-former orientation complementary to the binding pockets of the fibril. The order of the binding affinity of **109**, **118**, **119** and **120** are consistent with the order of their emission maxima bound to A β 1-42 fibrils, demonstrating that the effective conjugation length will affect the binding affinity.¹⁷⁵⁻¹⁷⁶

Finally, we investigated the binding specificity of **109** to **120** and BTDSB as well as DF-9 (control) by an *in vitro* fluorescence-based assay using APP23 transgenic mouse brain sections aged 19.6 months, an AD mouse model with later onset of amyloid plaque pathology than APP/PS1.¹⁷⁷ Old APP23 mice show a pronounced intrinsic amyloid plaque structural polymorphism likely associated with that APP23 mice generate a rather high A β 1-40/A β 1-42 ratio compared to that of APP/PS1 mice.¹⁷⁷⁻¹⁷⁸ We therefore selected this mouse model to assess the possibility of probing amyloid structural polymorphism by the ligands. As shown in Figure 4.10, fluorescence from the **109** bound to plaque core and coronal plaque can be easily discriminated by virtue of their spectra (Figure 4.10A and B), which can be used to monitor plaque maturation. What is the basis for this variation? Testing **109** in various solvents with decreasing dielectric constants reveals a linear dependence on the emission peak (Figure 4.9D). The slope was most pronounced for **109** and was less pronounced for **114** (data not shown) proposing that we actually probe variations in amyloid fibril binding site hydrophobicity by **109** and **114**.

In summary: These data extend the SAR of BTD-based A β binding and provide further direction for the development of noninvasive A β binding ligands for early detection of AD. The new bis-styrylbenzo-1,2,5-thiadiazole prototype could serve as a building block for the design and discovery of new early diagnosis for AD and potentially for AD therapeutics.

Figure 4.10 Confocal images of APP23 mouse (female, 19.5 months) brain sections (A) with accompanying normalized emission spectra of **109** (500 nM) bound to Aβ plaque core (black arrow) and plaque corona (red arrow). Scale bars = 50 μm. C) Chemical structure of **109**. D) Emission maxima dependent on polarity of **109** in different organic solvents (dioxane, toluene, EA, THF, acetone, DMF and DMSO) as well as in water.

5. Conclusions

In the thesis, my work has focused on the synthesis and characterization of fluorescent stilbene-based probes targeting amyloid fibrils. Among the studies, the following conclusions can be drawn:

- ❖ In general, we have displayed a general synthetic route, whereby a functional group unit was replaced with other functional motifs. This strategy can be used for synthesizing a selection of ligands that can be utilized for fluorescence detection of protein aggregates.
- ❖ Results from all the papers strongly demonstrated that analogous compounds exhibit similar binding properties but specific fluorescence properties.
- ❖ Pyrene derivatives are important organic chromophores with long excited state lifetimes when bound to amyloid fibrils. Pyrene derivatives involving substitution at the 1-position or substitution at the 2-position have distinct geometric, electronic structures and amyloid-binding properties.
- ❖ The X-34 scaffold, while rather well studied, may continue to serve a role for design of high-affinity and near infrared fluorescent (NIRF) ligands targeting A β and Tau pathology in human AD brain. Especially these ligands target A β pathology independently of the PiB binding site.
- ❖ Fluorescent small-molecule 2,1,3-benzothiadiazole (BTD) derivatives are a new class of bio-probes with attractive photophysical properties beyond the current classical scaffolds. This new class of bio-probes could be key player in the future of molecular probes, especially in the field of AD.

6. Future Perspectives

In this thesis, many intriguing conclusions have been drawn, hopefully, leading us one step closer to understand the relationship between the design of molecular probe and amyloid-binding properties, especially, the binding with A β plaques and tau tangles in AD. Therefore, there is still much interesting work to be done in the light of current findings.

Paper III shows four X-34 derivatives (NSB, TSB and QSB as well as BTDSB) with *in vitro* specific amyloid staining property of A β plaque and NFTs in human AD brain tissue except for QSB, which selectively and specifically only stained A β plaques. Especially, BTDSB displays emission in the red region (620 nm) with large Stokes shift (133 nm) and also high binding affinity (K_d : 93 ± 5 nM). Whether these ligands could stain A β plaques and NFTs *in vivo* using mouse or fly models of AD would be interesting.

Further analysis of the molecule in paper IV would be of interest. Probe **109** has specific staining properties in APP23 mouse brain sections (female, 19.5 months) and probe **120** has the strongest binding affinity among the probes as well as exhibits more than 650 nm emission maximum (in the NIR window from 650 – 900 nm) when bound to A β fibrils. But both probes have low solubility in water, especially **120**. We can modify their structures by linking a sugar or triethylene glycol (TEG) to the oxygen of the *para*-OH phenyl to increase their solubility. It would be interesting to investigate their two-photo emission and staining properties *in vitro* or *in vivo*. Relevant work is ongoing.

7. References

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