Evaluation of novel fluorescent probes for \textit{in vivo} Transthyretin amyloid using fibrils generated \textit{in vitro} under varying conditions

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Abstract
Transthyretin (TTR) amyloidosis is a disease that appears in three variants. One variant affects the elderly population with heart failure, the other two variants are hereditary and caused by an amino acid substitution in the gene, resulting in polyneuropathy and/or heart issues depending on the amino acid substitution. However, in all three variants, other organs may also be affected with amyloid deposition in the disease course. Amyloid fibrils of TTR (ATTR) contains a mixture of full-length protein and fragments (50-127).

Luminescent conjugated oligothiophenes (LCO’s) are novel amyloid binding probes used to stain amyloid fibrils and these amyloid probes have the feature of characterizing the amyloid structure in terms of fluorescence spectra. Apart from LCO’s, a few other amyloid binding probes are used to stain recombinant amyloid transthyretin and native transthyretin for binding studies.

The majority of generated TTR aggregates in vitro did not have the characteristic fluorescence spectra when bound to LCO’s and was observed as a clumped gel-like aggregate. The generation of recombinant TTR fibrils in vitro using the mutant TTR-T49M to obtain an aggregation prone fragment (50-127) after being treated with cyanogen bromide had a low yield of in vivo amyloid-like fibrils, but with characteristic LCO spectra.

Carpal tunnel ATTR often precedes ATTR deposition in heart tissue. Amyloid transthyretin in carpal tunnel tissues was stained with LCO’s and used as a reference in the comparison against the in vitro generated recombinant amyloid transthyretin fibrils. This project also includes quantification of amyloid transthyretin in a few selected parts of the carpal tunnel tissue using ImageJ. In the long run this method could help in diagnosing TTR amyloidosis.

Keyword
Transthyretin amyloidosis, Luminescent conjugated oligothiophene, Recombinant transthyretin fibrils, TTR-T49M, Cyanogen bromide, Fluorescence spectra
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Abbreviations

AD – Alzheimer’s disease
ATTR – Amyloid transthyretin
ATTRm – Mutated transthyretin amyloid
CIDP – Chronic inflammatory demyelinating polyneuropathy
CR – Congo Red
CSF – Cerebrospinal fluid
CV – Column volume
DTT – Dithiothreitol
DNA – Deoxyribonucleic acid
E. Coli – Escherichia coli
EDTA – Ethylenediaminetetraacetic acid
FAC – Familial amyloid cardiomyopathy
FAP – Familial amyloid polyneuropathy
LCO – Luminescent conjugated oligothiophene
h-FTAA – heptamer-formyl thiophene acetic acid
hx-FTAA – hexamer-formyl thiophene acetic acid
p-FTAA – pentamer-formyl thiophene acetic acid
IEC – Ion exchange chromatography
IPTG – Isopropyl β-D-1-thiogalactopyranoside
LB – Lysogeny broth
LNP – Lipid nanoparticles
LT – Liver transplantation
MW – Molecular weight
MWCO – Molecular weight cut-off
NSAID – Non-steroid anti-inflammatory drugs
OD600 – Optical density 600 nm
PBS – Phosphate buffered saline
pI – Isoelectric point
PPI – Proton pump inhibitor
RBP – Retinol binding protein
RCF – Relative centrifugal force
ROI – Region of interest
RPM – Revolutions per minute
SCA – Senile cardiac amyloidosis
SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC – Size exclusion chromatography
SOC – Super optimal broth with catabolite repression
SiRNA – Small interfering RNA
SNP – Single nucleotide polymorphism
SSA – Senile systemic amyloidosis
SUN – Spectral un-mixing
TBG – Thyroxine-binding globulin
ThT – Thioflavin T
TH – Thyroxine hormone
TTR - Transthyretin
T3 – L-3,5,3’-triiodothyronine
T4 – L-thyroxine
UV – Ultra violet
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1 Introduction

Transthyretin (TTR) is one of many amyloidotic proteins linked to the group of diseases known as amyloidosis. Amyloidosis is characterized by protein misfolding and formation of highly stable, insoluble and β-sheet rich amyloid fibrils in the body. The deposition of TTR fibrils in the extracellular tissues is the root of the diseases known as senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC).

Today, there are several treatments for TTR amyloidosis. Examples of treatment strategies are liver transplantation and pharmaceuticals that stabilizes the homotetramer. Inhibiting the production of TTR in the liver through gene silencing and degradation of TTR fibrils in vivo have recently been approved. These examples are therapies to prevent progression of TTR amyloidosis [1]. Even though no cure is available at present time, the research is moving forward, more knowledge is accessed through clinical trials, clinicians being more familiar with TTR amyloidosis and new research ongoing.

There is a broad range of biophysical tools and techniques that has been contributing in understanding the structure of amyloid fibrils at a molecular level such as nuclear magnetic resonance (NMR), Circular Dichroism (CD), X-ray fiber diffraction, atomic force and electron microscopy (AFM & EM) and a few other [2]. As for studies regarding amyloid formation kinetics, binding assays with Congo Red and Thioflavin T (CR and ThT) have been used for decades [3]. In pathology studies of amyloid fibrils ex vivo, CR has the property to specifically bind to amyloid fibrils which produces a green birefringence when exposed to polarized light using a light microscope [2]. More recent methods for characterization of amyloid structure with amyloid binding probes are the usage of Luminescent Conjugated Oligothiophenes (LCO’s) which are proven to have advantages over CR and ThT in terms of binding sensitivity and selectivity but also spectral characterization in fluorescence measurements [4,5].

Diagnosis of amyloid deposits in the extracellular compartment in our organs and tissues is of utmost importance. Tissue biopsy in combination with staining of fluorescent dyes is a very common and reliable diagnosis method for amyloidosis. There are, however, non-invasive methods for diagnosis of cardiac amyloidosis such as electrocardiography and cardiac magnetic resonance (CMR). The diagnosis of TTR amyloidosis remains a challenge, many non-specific symptoms linked to ATTR may resemble other types of diseases and can lead to misdiagnosis. The fact that therapies vary between different kind of amyloidosis and may associate with toxicity upon mistreatments, makes it important to ascertain the protein responsible for the disease. Identification of responsible protein may be performed with immunohistochemistry, electron microscopy or mass spectrometry [6].

1.1 Background and aim of the project

The aim of this project is to acquire more knowledge of TTR fibrils, including how native TTR interacts with several different amyloid binding probes. Apart from previous studies on h-FTAA and p-FTAA with TTR fibrils in vitro and in human tissues, other comparable probes, will be tested in this project. Reliable amyloid binding probes is of great importance for detecting amyloid depositions in the human body as early as possible. This paves the way for treatment early in the disease course.
This project consists of three studies, a study based on binding and affinity measurements of amyloid probes to native TTRwt and fibrils. Amongst the amyloid binding probes used in this project, three belong to the group called LCO’s which includes p-FTAA, hx-FTAA, h-FTAA [7]. These probes are characterized by a varying number of thiophene’s in the backbone of the molecule. Two other thiophene-based probes HS-169 [8] and an unpublished amyloid probe HS-199 from Linköping’s university. Two conventional amyloid probes, CR and ThT as well as the CR analogue BTDSB [9] will also be included in the project. The usage of LCO’s for binding amyloid fibrils has a favourable trait, where two characteristic peaks are seen in the emission spectrum.

The second study is to attempt on in vitro generation of TTR fibrils which bind fluorescent dyes resulting in spectral properties with hope to be similar to those seen in tissues. TTR is one of the extraordinary amyloidotic proteins where most of the formed fibrils in vitro are structurally different to those seen in vivo. It would be beneficial for research purposes to form in vivo-like TTR fibrils through in vitro methods. Common strategies for fibril formation will be used, such as specific environmental conditions (pH and temperature). A mutation of the TTR gene will be implemented resulting in an amino acid substitution (T49M) to enable truncated TTR fragments (50-127). This fragment is known to have an increased rate of fibrillogenesis and that has been found in human tissues related to cardiac amyloidosis [1,10]. Cyanogen bromide (CNBr) will be used in attempt to obtain the amyloidogenic fragment through C-terminal cleavage at the introduced methionine.

In the last study, TTR amyloid quantification of carpal tunnel tissues from a Japanese patient will be performed using the amyloid probes hx-FTAA and h-FTAA. Fluorescence microscopy will be used to acquire spectral images, and for the quantification, an image processing program known as ImageJ is used.

2 Process
2.1 Project plan
This project was carried out at Hammarström Lab in Linköping’s University for 21 weeks. At the beginning of the project, an initial GANTT-chart was established, where all planned activities were introduced in a chronological order. With the help from my examiner and supervisor, an approximate time for each experimental procedure was determined, see Figure 1.

The first main objective was to achieve enough purified TTRwt to start the amyloid fibrillation experiments. One week would normally be sufficient for protein purification, but because of the ÄKTA-system being completely unfamiliar to me, two weeks was set. The second main objective was reached after performing planned fibrillation experiments and fluorescence measurements of amyloid binding probes to TTRwt fibrils with the fluorescence plate reader. The results were to be presented during the half period project meeting, to further discuss whether adjustments of WT experiments were required otherwise planning of the T49M mutant experiments could be made. To reach the final main objective, all experimental parts of the project had to be completed and the final submission of the project
2.2 Process analysis

The main priority of the laboratory work, at the start of the project was to obtain purified TTR^wt to start the first study of this project, generating recombinant TTR^wt fibrils. At the second purification attempt, enough WT TTR was obtained. The generation of recombinant TTR fibrils followed standard fibrillation methods. As expected, it was not easy to obtain recombinant TTR fibrils similar to those seen in tissues containing TTR amyloidosis. This was confirmed by the emission spectra, when staining the fibrils with LCO’s and analysing with both fluorescence plate reader and fluorescence microscopy. The fluorescence measurements started earlier and lasted longer than anticipated. This was due to set up adjustments and mixing of new amyloid probes causing experiments to be redone. The experiments using fluorescence microscopy required one additional week, since practice is essential when searching for and distinguishing amyloid fibrils from other auto fluorescent molecules. Since it could be a lot of waiting time between experiments, procedures with TTR-T49M were performed in parallel to the ex vivo tissue experiments and the quantification of amyloid in carpal tunnel tissue.

Learning the ÄKTA-system was quite complex at the beginning and the introduction of the system was mostly about how to avoid damaging the system, how the fluid went through the system and in what order tubes had to be connected. Through trials, it took about one week to learn it in a manner where it felt confident to run the procedures manually. Understanding how to set up methods for Ion-Exchange Chromatography (IEC) and Size Exclusion Chromatography (SEC) took slightly longer and help was provided by my examiner and another master thesis student.

It was planned to use a software known as Aqua to quantify amyloid deposits in carpal tunnel tissues. However, due to a bug in the software code, the software crashed when starting any measurement. As a plan B, provided by my examiner, the image processing software ImageJ would be used for the quantification. How the software would be used to quantify the spectral images were not fully known. After some information searching and discussion with my examiner and supervisor, they recommended using the “colour threshold” function and a plugin known as “Colour Pixel Counter”.

Figure 1. GANTT-chart shows the timeline of activities planned at the beginning of the project. Pink bars indicate project start-up activities. Green bars correspond to laboratory related activities. Red bars correspond to report related activities.
The project process advanced satisfactorily (see Figure 2) where all implemented activities from the initial plan was performed within time frame and the obtained results to a degree where the aim of the project was achieved and hopefully in contribution to further related studies.

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Figure 2. GANTT-chart showing the actual progression of the activities during the whole project.

3 Theory

3.1 Amyloidosis

The term amyloidosis is classified as a group of diseases in which normal soluble proteins are triggered to undertake conformational changes that causes the protein to aggregate and form toxic amyloid fibrils. A common triggering effect that stimulates protein unfolding and eventually misfolding, is destabilization of the protein’s native structure and can be due to mutations and changes in the physiological conditions. Accumulation of amyloid fibrils in the extracellular tissues is the progression of amyloidosis. If left untreated, these types of diseases lead to death through tissue damage and organ dysfunctions causing heart failure, polyneuropathy and dementia, depending on what protein or peptide and where the amyloid fibrils are located [11].

3.2 Amyloid formation

It is widely acknowledged that the formation of amyloid fibrils is induced by a conformational change or a peptide cleavage to facilitate a conversion of a normal soluble protein into an unstable conformational structure allowing the protein to self-assemble into amyloid fibrils. In vitro amyloid formation can be induced by exposing the protein to partially denaturing conditions or by adding structured fibril aggregates known as “seeds” to a solution of soluble precursor protein of the same sequence. Proteins adopting alternative conformational intermediates that are in thermodynamically unfavourable states are susceptible to rapidly develop into amyloidogenic forms [2,3,12].

Amyloid fibrils may or may not be physically, morphologically or tinctorial similar. In the case of Aβ 1-42 which is related to Alzheimer’s disease (AD) the in vitro produced fibril structures looked similar to those seen in vivo [2], whilst the majority of in vitro TTR generated fibrils are rather different to those deposited in vivo.
3.3 Functional amyloid
The existence of non-pathogenic and functional amyloid has been discovered in bacteria, fungi, insects and mammals. A type of fibre known as Curli is formed by a protein called CsgA, those fibres have been found on the surface of E. coli which has a similar structure to amyloid. Filamentous fungi produce hydrophobins which may assemble to fibrils with a function believed to protect its structure. The protein Pmel17 is involved in biosynthesis of melanin in mammals. Pmel17 forms fibrillar structures with similar characteristics to amyloid fibrils. One of the reasons why amyloid fibrils being destructive when accumulated in tissues is their highly ordered, insoluble and stable structures [2].

3.4 Transthyretin

3.4.1 Transport protein
TTR is a transport protein and is classified as one of the three foremost thyroid hormone-binding proteins in the plasma and the major thyroid hormone (TH) transport protein in cerebrospinal fluid in human. It also participates in transporting retinol (vitamin A) by binding to a retinol-binding protein. TTR binds both forms of thyroid hormones L-3,5,3’-triiodothyronine (T3) and L-thyroxine (T4), however, mainly the latter. Being one of the three major TH binding proteins along with albumin and thyroxine-binding globulin (TBG), the task is to ensure an appropriate distribution of TH in the body as well as maintaining a free hormone pool in the blood and CSF. Due to TTR having an association and dissociation rate between albumin and TBG it is considered to be the most important T4 distributor in human [13]. Tetrameric TTR has two iodothyronine binding sites. TTR commonly binds only one T4 molecule under physiological conditions due to negative cooperativity effect, which greatly reduces the binding affinity on the other site after one T4 molecule binds to the first site. One TTR tetramer can bind up to four RBP molecules along with one T4 molecule [14]. TTR is dominantly synthesized in the liver and secreted into the blood plasma but is also produced by the retinal pigment epithelium and the epithelial cells of the choroid plexus in the brain which is secreted into the eye and CSF, respectively [15].

3.4.2 Structure and evolution
Human TTR is encoded by a single gene located in the X-chromosome (Xq22.2). The gene is built up from 5 exons covering totally 5.5 kbp [14]. Monomeric TTR in human consists of 127 amino acids but may vary between 125-136 depending on which vertebrae species it is derived from. TTR mainly exists as a homotetramer in vivo with a molecular weight of 55 kDa. The monomeric subunits comprise of 8 β-strands forming 2 β-sheets and a small α-helix of 9 amino acid residues. The four subunits of the tetramer form a β-barrel structure. TTR is a highly stable protein and the high β-sheet content is believed to contribute to its stability [13].

The primary structure of monomeric TTR has been analysed in more than 30 animal species and contains two parts, a pre-segment necessary for extracellular secretion and a mature polypeptide segment essential for the functioning of the protein. Prapunpoj et al. aligned 25 vertebræ species of TTR amino acid sequence including human TTR and showed that the amino acids in the central channel, involved in the binding interaction to THs, were conserved and had not been altered for many million years [13]. Most evolutionary changes occurred in the N-terminal section of the monomer. The binding affinity of TTR to thyroid hormone T4 is dependent on the N-terminal length and the number of hydrophilic groups located in the N-terminal. However, the evolutionary changes on the C-terminal region has
occurred less in comparison to the N-terminal of TTR. The slight differences in human TTR compared to other vertebrates species are the amount of hydrophobic amino acid in the C-terminal region. The influence of accessibility to TH binding sites may depend on the C-terminal region due to the fact that the segments are close to the central channel where the binding sites exist [13], see Figure 3B. The C-terminal peptide fragments (starting at position 46-52) are known to be linked to the pathogenesis of SSA since the aggregation of TTR is strongly due to the rigid properties of the C-terminal region [1].

![Figure 3. Generated in PyMOL A) Monomeric structure of TTR, N-terminal (blue) and C-terminal (red), PDB ID: 1BMZ. B) Showing a TTR dimer-dimer interaction that forms a TTR tetramer. The black arrow in the middle shows the central channel where two thyroxine binding sites are located, shown in dotted squares, PDB ID: 1ICT.](image)

3.5 Diseases linked to TTR

3.5.1 Senile systemic amyloidosis

The age-related amyloid disease known as SSA due to amyloid deposition in organs and tissues was defined in 1876 by Soyka [16,17]. SSA, previously called senile cardiac amyloidosis (SCA) is originated by wild type TTR which has no genetic variation and is prone to affect the elderly population with heart failure. The occurrence of SSA is increasing with age, and patients mainly diagnosed with amyloid deposits in various tissues related to SSA are over 60 years old. In SSA the amyloid fibrils are primarily concentrated in the heart, however, it can also be systemically located in many other organs and tissues [18]. There are assumptions of SSA being gender-specific, this disease is reported to be predominant in males compared to females, especially when it comes to amyloid deposit load in tissues [17].

3.5.2 Familial amyloid polyneuropathy

In 1952, a physician named Corino Andrade was the first to report on the disease we today call amyloid transthyretin familial amyloid polyneuropathy (ATTR FAP), he described it as “a peculiar form of peripheral neuropathy” [19]. FAP is an autosomal dominant and adult onset disease characterized by amyloid deposits in the peripheral nerves causing irreversible, progressive and persistent nerve damage [15]. FAP patients may involve with or without cardiac amyloidosis [17]. The most frequent mutation associated with FAP is V30M and is responsible for 50% of the ATTR FAP mutations cases over the world [20].

In early onset of ATTR FAP which have been found in endemic regions (Portugal, Cyprus, Japan and Majorca), the symptoms arise before the age of 40 with an average life expectancy
of 10.8 years at disease onset. The symptoms are progressive sensorimotor and autonomic neuropathy, starting with disturbance in sensing the toes and feet rising to the legs and will eventually develop in the hands, also involving loss of thermal sensation. In non-endemic parts and the ATTR FAP endemic part of northern Sweden, in an area known as Skellefteå, the onset of ATTR FAP usually occurs after 50 years of age, classified as late onset. The late onset variant typically has a more severe disease course but a lower penetrance rate starting with sensorimotor symptoms from the hip to the toes and eventually causing loss of all sensory modalities and may resemble chronic inflammatory demyelinating polyneuropathy (CIDP), as a result of degeneration of the myelin sheath of peripheral nerves. The common course of ATTR FAP can be divided into three stages; sensory polyneuropathy, progressive walking incapacity and wheelchair bound [15,20].

The fact that ATTR FAP can be involved in many other tissues and organs such as the heart, gastrointestinal tract and carpal ligament makes diagnosis of ATTR FAP yet a challenge. The genetic variability and non-specific symptoms generally lead to delay in diagnosis and even misdiagnosis [15,20].

3.5.3 Familial amyloid cardiomyopathy
Familial amyloid cardiomyopathy is a variant of TTR amyloidosis (like FAP) caused by a single point mutation in the TTR gene. FAC is characterized by amyloid deposition in the heart, resulting in heart failure, dissimilar to FAP that is more commonly involved with amyloid deposits in the peripheral nerves [17]. The single nucleotide polymorphisms (SNPs) phenomenon is when a single DNA building block, so called nucleotide, is replaced with another nucleotide. This phenomenon is the most common way of generating genetic variations in the genes. In fact, the change alters the gene to produce a variant of the protein that may have a direct role in progressing a disease [21]. There are more than 100 SNPs encoding alternates of TTR and with 80 mutations confirmed to be associated with pathogenesis. The most predominant mutation of TTR variant targeting the heart is TTR-V122I [22].

TTR-V122I is known to alter the equilibrium of tetrameric TTR towards monomer leading to tetramer destabilization. This variant is also lowering the kinetic barrier which is related to the rate-limiting tetramer dissociation causing more rapid and frequent release of folded monomeric TTR, essential for amyloid fibril formation. The formation of folded monomeric TTR rapidly undergo partial denaturation and assembles into amyloid fibrils. Jiang et al. compared the dissociation rate of tetrameric TTRwt and TTR-V122I into monomers and the fibril formation rate in vitro. V122I forms TTR fibrils twice as fast from tetramers compared to WT implying that V122I variant is less stable than TTRwt [22]. Back in 1996, Jacobson et al, reported that the V122I was estimated to be heterozygous in 3-4 % of the African American population, thus, being at risk in developing ATTR cardiac amyloidosis [17].

3.6 Treatments for TTR amyloidosis
Currently, there is no cure for individuals affected by TTR amyloidosis, neither for the inherited variants or SSA. However, there are treatments against TTR amyloidosis. Liver transplantation (LT) is a way to halt the production of mutated TTR variants, thus preventing amyloid formation caused by mutated TTR, nonetheless the patient may develop amyloidotic symptoms related to SSA. Patients with potential risk of developing severe sensorimotor
neuropathy in the lower extremity or having amyloid involvement in multiple organs such as in the gastrointestinal tract and heart are usually those having liver transplantation [23].

Various of studies on how the formation of TTR amyloidosis occurred, led to the concept that TTR tetramer stabilization would prevent amyloidogenesis. It is known that tetrameric TTR alone is not amyloidogenic, however, when a tetrameric TTR dissociates into monomers, the monomers may become non-native with low conformational stability and drastically increase the rate of amyloid fibril formation [1].

Contemporary, there are a few TTR stabilizing pharmaceuticals available such as tafamidis and diflunisal. Diflunisal is a nonsteroidal anti-inflammatory drug (NSAID) that reduces the rate of neurological impairment progression in patients suffering from FAP. Diflunisal stabilizes familial ATTRm variants by binding to the thyroxine binding sites of TTR which prevents acid mediated fibril formation. Unfortunately, diflunisal has the consequence of interacting with other cellular receptors and enzymes in ATTR patients with cardiac amyloidosis and may cause gastrointestinal bleeding, renal dysfunction, fluid retention and hypertension in more susceptible individuals. As a compensation for ATTR patients with cardiac amyloidosis, a cohort study suggested low dosage of diflunisal with a prophylactic proton pump inhibitor (PPI) could allow safe administration [1,23].

Tafamidis is a drug that like diflunisal binds to thyroxine-binding sites in the TTR tetramer preventing its dissociation into monomers, which stops the amyloidogenesis. In clinical trials, it has been proven that tafamidis has stabilized TTR tetramers for patients with early-stage V30M variant, but also for patients suffering from cardiac and neurological ATTR without V30M and V122I [23]. In a phase 3 trial, the outcome of the treatment of TTR cardiomyopathy with tafamidis was positive, tafamidis reduced mortality and cardiovascular hospitalizations [24]. The US Food and Drug Administration (FDA) has recently approved tafamidis as a treatment for TTR cardiac amyloidosis [25].

Other ongoing methods to prevent TTR amyloidosis and amyloidosis in general are silencers. The strategy of using small interfering RNAs (siRNAs) to suppress the expression of TTR in hepatocytes through cleavage of TTR mRNA is promising. The anti-transthryretin agents ALN-TTR01 and ALN-TTR02 are lipid nanoparticles (LNP) consisting of siRNAs targeting a sequence conserved in the untranslated 3’ region for both wild-type and mutant TTR mRNA. These agents deliver the siRNA to hepatocytes and suppresses the expression of TTR. Reduction of amyloid precursor protein production is of great importance in treating amyloidosis, however, the capability to eliminate deposited amyloid fibrils in tissues is likely an important step to advance forward in curing amyloidosis [1,23].

3.7 Probes used for staining ATTR

Small fluorescent molecules of the group luminescent conjugated oligothiophenes are powerful tools used to stain protein aggregates. Compared to the conventional amyloid binding probes such as CR and ThT, LCO’s have been proved to detect a higher variety of protein aggregates associated with disease. LCO’s with at least 5 thiophenes in their backbone have the capability to display characteristic fluorescence spectra in terms of curve shape or double peaks at specific wavelengths when bound to the repetitive β-pleated structure, such as the structure of amyloid fibrils [8,26]. This characteristic feature changes depending on the locked angle of the thiophene backbone when bound, generating structural information. In this regard, it is possible to distinguish between proteins and their alternate
forms of protein aggregations such as fibril structure or other morphological structures [27]. LCO’s have advantages compared to CR staining in form of detection sensitivity to premature fibrils, it has been shown that LCO staining with h-FTAA in carpal tunnel tissues have been amyloid positive that has been negative for Congo Red [28]. ThT and Congo Red can only declare amyloid positive or negative.

BTDSB is a CR analogue synthesized by Zhang et al. to image and study Aβ and Tau fibrils which are the hallmarks for Alzheimer’s disease [9].

HS-169 is a pentameric oligothiophene derivative with a negative charge. This thiophene-based ligand was synthesized to detect protein aggregates while covering a broader emission range in the visual spectrum with hope of combining LCO’s with fluorophore labelled antibodies to selectively detect proteins [8].

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**Figure 4.** The molecular structures of three LCO’s. A) Pentamer-formyl thiophene acetic acid. B) Hexamer-formyl thiophene acetic acid. C) Heptamer-formyl thiophene acetic acid.

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**Figure 5.** Molecular structure of the amyloid binding probes BTDSB and HS-169.
3.8 Protein purification

3.8.1 Ion-exchange chromatography

Ion exchange chromatography (IEC) is a purification method separating molecules based on their net surface charge. The molecules are for example oligonucleotides, proteins, peptides or other charged molecules. An ion exchange column comprises of certain types of resins, that contains either positively charged functional groups (anion exchange) or negatively charged functional groups (cation exchange). The choice of exchanger to be used depends on the charge of the protein, the protein must have an opposite charge to the functional groups of the exchanger in order to bind to the resin. In IEC, manipulation of interactions between charged molecules and opposite charged functional groups of the resin is performed to favour binding or elution of molecules. A molecule, such as a protein does not have a net charge at a pH corresponding to its isoelectric point (pI), thus will not bind the functional groups on the resin. However, at a pH below its pI the protein binds to a negatively charged functional group (cation exchanger) and at a pH above its pI the protein binds to a positively charged functional group (anion exchanger). The occurrence of other types of bindings apart from the ion exchange interaction may be common, such as van der Waals forces and nonpolar interactions but has a very small effect on the separation. IEC can be divided into 4 steps; equilibration, sample application and wash, elution and regeneration [29].

Equilibration of the column resin is performed to reach a desired start condition. That is when the resin or so-called stationary phase have their functional groups bound to counterions, such as chloride or sodium. The start buffer used for equilibration is selected to certify that the sample containing the protein of interest will bind to the resin when being loaded [29].

When applying the sample and binding it to the resin, washing is performed to get rid of unbound compounds, that is molecules with opposite charge contra the functional groups in the resin or molecules with the same charge. It is important that the sample buffer has the same ionic strength and pH as the starting buffer to bind the majority of the protein of interest [29].

In the elution step when the sample has passed through the column and most impurities been washed away, elution of the protein of interest is performed. The elution is done by either increasing the ionic strength or by changing the pH. By increasing the ionic strength of the buffer, the salt ions such as Na⁺ or Cl⁻ competes in the binding to the functional groups. The increase in ionic strength will cause the proteins with lowest net charge at the selected pH to elute first. Proteins with highest net charge at the selected pH will elute last and require high ionic strength to be eluted [29].

To ensure a good performance by the column in the next run, a final wash with high concentration of salt is performed to remove any molecules still attached to the resin [29].

3.8.2 Size exclusion chromatography

Size exclusion chromatography (SEC), particularly gel filtration is a method to separate compounds based on the difference of their hydrodynamic size using aqueous solvent. SEC is commonly used for purification of proteins and for protein separation but can be applied to separate nucleic acids, polysaccharides and other biological molecules. In SEC, the compounds do not bind to the column as for IEC, instead they pass through a gel filtration medium consisting of
pores of different sizes, that smaller compounds will enter, thus, delaying the elution time while the larger compounds too large to enter the pores will pass through the column and elute first. This method can be used along with a UV-light detector to record peaks, notifying when compounds absorbing, and emitting light are being eluted, specifically proteins [30].

SEC is the mildest of all chromatography methods, it is well suited for biomolecules sensitive to changes in pH, concentration of metal ions or co-factors. The separation can be performed in the presence of different molecules such as detergents, urea, guanidine or cofactors [30].

3.8.3 Dialysis
Dialysis is a technique performed to remove unwanted compounds such as salt, detergents and small molecules from a sample solution. Molecules smaller than the membrane-pore size of the dialysis tube will passively diffuse through and reduce the amount of contaminants in the sample. Larger molecules will remain in the sample solution, thus allowing removal of unwanted compounds in a sample solution to a negligible level [31]

3.8.4 SDS-PAGE
Protein applications with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) allows verification of the protein of interest and a positive/negative protein presence. SDS denatures and interacts with the hydrophobic regions of the protein, presenting it with an overall negative charge and a consistency in protein shape. This allows the protein to migrate size dependently, thus, larger molecules with a higher molecular weight will be retained and travel a shorter distance, time wise, compared to smaller molecules. A ladder with known molecular weights is used as a comparable source for the protein sample of interest [32]. SDS-PAGE may even be used as a controlling factor for detection of protein aggregations [33].

3.9 Fluorescence spectroscopy
Fluorescence is a phenomenon that occurs after a molecule is excited from the ground energy state to an electronic excited state (higher energy state) by absorbing light (photons). There are different vibrational levels in the electronic excited states, any excess of energy in the excited molecule after reaching the first electronic excited state is converted to vibrational energy, and the molecule may end up in higher vibrational levels or even higher electronic states, depending on the photon energy absorbed. Upon collision with solvent molecules the energy of the excited molecule is converted to other forms of energies and the molecule returns to the vibrational ground level of the electronic excited state. Solely, from the lowest level of the electronic excited state can the molecule transition back to the ground state by emitting light (fluorescence), see Figure 6. However, transition back to the ground state also occurs through non-radiative internal conversion, meaning that a transition occurs from a higher energy state to a lower energy state without emitting fluorescence. This arises when the vibrational levels of the ground states overlap with the vibrational levels of the electronic excited state [34].
Fluorescence spectroscopy is widely applied to studies in the chemical and biological sciences. Briefly, fluorescence spectroscopy involves using a beam of light of a specific wavelength, usually UV-light, that passes through a solution consisting of fluorophores absorbing and emitting light towards a filter and into a detector that measures the fluorescence. It is important to mention that radiative energy loss occurs in fluorescence compared to the absorption, thus why the emitting light usually has a longer wavelength and a lower energy than the exciting light. This is known as Stokes shift. Fluorescence spectrometers may operate by filters used to select specific wavelengths for excitation and emission or by monochromators, which are optical devices transmitting a specific band of the electromagnetic spectrum. Since one monochromator tunes the excitation wavelength whereas the other one analyses emission wavelength of the fluorophore, two types of spectra: excitation and emission spectra can be measured. To measure fluorescence excitation spectrum, the emission monochromator is set to a fixed wavelength ($\lambda_{em}$) while a preset range of the excitation wavelengths are being scanned. For measurement of emission spectra, a fixed excitation wavelength ($\lambda_{exc}$) is set, and a scanning of a preset wavelength range with the emission monochromator is performed [35].

3.10 Fluorescence microscopy

Fluorescence microscopy is referred to all microscope instruments using fluorescence to image objects of interest. There is the conventional widefield fluorescence microscope with a
less complex set up or a confocal microscope with an upgraded design. The general principle of the fluorescence microscope is the usage of a light source sending out light of different wavelengths, from UV to infrared, that passes an excitation filter which filters all wavelengths of light except for one specifically selected. The selected wavelength of light is reflected by a dichroic mirror towards the objective lens to the specimen, the excited fluorophores will then emit light of a higher wavelength that passes through the objective and the dichroic mirror without being reflected and towards a barrier filter known as the emission filter. The emission filter allows passage of only longer wavelengths to the detector. A confocal microscope has the trait of scanning an image, this means that one region of the specimen is sampled at a time, basically one fragment of the image is obtained per scan and the fragments from previously scanned regions are added together, like a puzzle. Scan one provides with one fragment, scan two with the second fragment, and it keeps on building until the whole selected field of view is sampled and an image is built up. The confocal microscope scans the image by illuminating each individual region in sequence and blocks all light except for the illuminated regions from returning light to the detector with the usage of pin-holes. Since the fluorophores emit light in all directions, only light emitted from a certain focal point in the specimen passes through the pin-holes to the detector. A confocal microscope eliminates background caused by scatter and out-of-focus light, resulting in higher resolution images. However, one drawback is the image acquisition not being as rapid as the widefield fluorescence microscope, due to many measurements being taken. In widefield fluorescence microscopy, the whole focal plane is simultaneously illuminated by light resulting in a lower contrast image [36].

Fluorescence microscopy is widely used in biology and medicine using fluorescently labelled antibodies or intrinsically fluorescent reporter proteins, but also in combination with fluorescent probes for e.g. amyloid. The usage of fluorescence microscopy requires that the object of interest fluorescence, thus why usage of fluorescent probes are important when studying tissues afflicted by disease or just for studying cellular processes [37].

4 Materials and Methods
4.1 Methods – Transformation, mutagenesis, expression and extraction of TTR
4.1.1 Transformation
Electrocompetent *Escherichia Coli* (E. coli) of strain BL21(DE3) stored in -80° C were thawed and 50 µl E. coli was mixed with 1 µl plasmid previously stored in -20° C containing the TTR gene of interest. The plasmid also contains a gene for antibiotics resistance, in this case ampicillin. The transformation of E. coli cells was performed using electroporation with MicroPulser Bio-Rad at 2.5mV. Directly after electroporation, 250 µl of prewarmed (37 °C) SOC medium was added into the electroporation cuvette, then transferred to a 1.5 ml microtube and incubated at 37° C for an hour. After incubation, the E. coli mixed with plasmid was spread over LB agar plates containing 1 mM ampicillin (100 µg/ml) to ensure that only bacteria successful in the transformation could grow on the plates. Overnight incubation was done in 37° C.
4.1.2 Site-specific-mutagenesis

LB media had a final concentration of 1 mM ampicillin in all steps.

The primers for T49M and S52P mutations were designed and ordered by my supervisor Sofie Nyström. GeneAmp PCR System 9600 (Applied Biosystems) and Q5 Site-Directed Mutagenesis Kit was used to perform the mutagenesis along with designed primers and plasmid containing TTR gene. The protocol was followed step by step from PCR to transformation [38], see Figure 7. One colony from T49M and S52P were each added into separate 10 ml LB broth and incubated at 37° C for approximately 6 hours before harvesting. QIAprep Spin miniprep Kit was used to harvest and purify the plasmids before sending them to GATC Biotech for sequencing.

![Figure 7. Overview of Q5 site-specific mutagenesis. Designed primers are required. (\*) indicate the incorporated nucleotide change(s) in the forward primer (black). Reverse primer (red) is designed in a way where 5' ends of the two primers anneal back-to-back. First step includes an exponential amplification using designed primers and a master mix of DNA polymerase. In the second step an incubation with an enzyme mix containing kinase, ligase and DPN1. Together these enzymes grant rapid circulation of the PCR product and removal of template DNA. Last step includes a high-efficiency transformation. For further information see Q5-site-specific mutagenesis kit instruction manual, Figure taken from [38].](image)

4.1.3 Protein expression

A small-scale protein expression control was made by adding one single colony of E. coli BL21(DE3) to 5 ml LB-media containing 100 µg/ml ampicillin and incubated at 37° C with shake (185 RPM). When OD₆₀₀ reached 0.4-0.6 the suspension was induced with IPTG to a final concentration of 1 mM and incubated for another 4 hours. The cells were harvested by centrifugation at 3000 RPM for 15 minutes at 4° C and resuspended in B-Per to lyse the cells before centrifuging again. The supernatant was collected and used for protein verification with SDS-PAGE.

After verification of correct expression of protein by SDS-PAGE, a large-scale protein expression was proceeded. A few colonies were collected with an inoculating needle and plated on new agar plates containing 100 µg/ml ampicillin. The plates were stored in 37° C overnight. In each agar plate, a few ml of LB-medium was added to suspend the bacteria colonies and poured into 2x 1.5 L LB-medium (3 L flasks). Each flask was supplied with 100 µg/ml ampicillin to a final concentration of 1 mM and incubated in 37° C with shaking (185
RPM). The OD\textsubscript{600} was measured with an OD\textsubscript{600} spectrophotometer from MIDSCI and once the bacterial growth reached OD 0.6, IPTG was added to each flask to a final concentration of 1 mM. The continuous bacterial growth after induction occurred in room temperature overnight.

4.1.4 Protein extraction
Bacterial cells were harvested through centrifugation at 4000 RPM for 30 minutes at 4°C and resuspended in 50 ml distilled water. The tubes containing bacteria diluted in distilled water were frozen in liquid nitrogen and stored in -80°C until needed. The lysis of the cells was done by firstly having the cells resuspended in distilled water, then freezing it and thawing it and finally through sonication on ice. The sonication was done with an amplitude of 30%, 5 seconds on time and 20 seconds rest, with a total time of 5 minutes (in total 12 times of 5 seconds bursts). To remove the cellular debris, the suspension was centrifuged at 20000 RCF for 30 minutes at 4°C. The supernatant was heated at 65°C for 30 minutes to precipitate unwanted proteins and centrifuged again. Lastly, the supernatant was filtered with a 0.22 µm filter.

4.2 Methods - Purification of TTR
Purification of TTR described in the two following sections were made with an automatic liquid chromatography system – ÄKTA 25M Pure with UV detector and fraction collector. UNICORN 7.0.1, a software provided by GE Healthcare was used to run the system and analyse the results.

4.2.1 Ion-exchange chromatography with Source Q
The Source Q column was thoroughly washed according to GE-healthcare Ion-exchange chromatography handbook and equilibrated accordingly, see table 1. The prepared protein sample described in the protein extraction section was manually loaded into the column with a syringe before using the ÄKTA system. A constant flowrate of 2 ml/min and a pressure alarm of 0.7 MPa was used during the entire purification and fractions of 5 ml were collected. UV detection at 280 nm (tryptophan and tyrosine absorption) was monitored to know when the proteins were eluted. A gradient elution was set from 0-100% with 1M NaCl 20mM Tris, pH 8 (buffer B) and fractionation started after washing with 2 CV of 100 mM NaCl 20 mM Tris, pH 8 (buffer A).

<table>
<thead>
<tr>
<th>Ion-exchange column wash</th>
<th>CV</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NaCl</td>
<td>2</td>
<td>2 M</td>
</tr>
<tr>
<td>2. NaOH</td>
<td>4</td>
<td>1 M</td>
</tr>
<tr>
<td>3. NaCl</td>
<td>2</td>
<td>2 M</td>
</tr>
<tr>
<td>4. MQ-H\textsubscript{2}O</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>5. Equilibration buffer /start buffer</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

The total time of fractionation was 10 CV (1 CV ~ 40 ml), TTR eluted at a gradient strength of 25-30%. The collected fractions were stored in 4°C and suspected fractions containing TTR were run on SDS-PAGE.

4.2.2 Size exclusion chromatography with Superdex 75 16/60
Fractions containing TTR verified from the SDS-PAGE were pooled together and concentrated. Superdex 75 16/60 column was equilibrated with PBS, before loading and injecting a syringe containing 1 ml concentrated protein sample into the system through a 1
ml loop. The SEC was run in 1 ml/min with a pressure limit on 0.6 MPa. Fractions of 3 ml were collected and TTR eluted after 50 ml which was seen in the chromatogram (Figure 8). Fractions containing purified TTR according to the chromatogram were run on SDS-PAGE.

---

**Figure 8.** Chromatograms after running SEC with the ÄKTA-system. Top chromatogram shows the purification of TTRwt and the bottom chromatogram shows the purification of TTR-T49M. Fraction B8-B5 contained TTRwt, fraction B11-B7 contained TTR-T49M. Blue, pink and red line indicates UV_{280}, UV_{254} and UV_{215} respectively. The purification of TTR-T49M was stopped earlier compared to TTRwt, since all TTR had eluted after ~ 60 ml. Pooled fractions of TTRwt and TTR-T49M from 1 ml IEC sample yielded in 94 μM and 72 μM, respectively, measured with fluorescence spectrometer Hitachi 2001 at absorbance 280 nm. SDS-gel of pooled fractions containing of both forms can be seen in Figure 24, well 2 and 7.
4.2.3 Concentrating TTR
To concentrate TTR protein, a Centriprep Centrifugal filter device with Ultracel 10K membrane was used. The fractions were added into the Centriprep tube and centrifuged 3 times using 3000 RCF for 15 minutes at 4° C. After usage, the Centriprep tube was rinsed, stored in distilled water in the refrigerator at 4° C and was reused.

4.2.4 Dialysis of TTR
Fractions containing TTR after SEC were pooled together. Half of the TTR was stored in 4° C as native tetramers and the other half was dialysed at 4° C using Spectra/Por Membrane tubing with a molecule weight cut-off (MWCO) on 12-14 kDa, over 48 hours. Dialysis was performed to obtain unfolded TTR. The first dialysis liquid was water for desalting purposes. This was followed by 10 mM HCl (pH 2) to unfold the TTR. After dialysis the unfolded TTR was kept in 4° C. Unfolded TTR tetramers were to be used in fibrillogenic experiments in vitro.

4.3 Methods - Protein analysis and fibrillation

4.3.1 SDS-PAGE
SDS-PAGE analysis was used for protein verification purposes and as a control regarding the purity of the protein of interest after the purification steps. 20 µl protein sample were mixed with 6 µl 4x SDS cocktail with DTT (loading dye). Additionally, 1 µl β-mercaptoethanol was added to ensure cleavage of disulfide bonds. The samples were heated at 95° C for 5 minutes before being loaded into the wells. The molecular weight marker Precision Plus Protein Dual Colour Standards (10-250 kDa) from Bio-rad was used, 5 µl was loaded into each used gel. To obtain optimal separation at 100 V, the running time was set to 80-90 minutes. When the electrophoresis was finished, the acrylamide gels were washed in distilled water 3x for 5 minutes and stained with Coomassie blue G-250 for 10-30 minutes. Destaining was performed by first heating the gel covered in destaining solution in the microwave for 20 seconds (800 W) and left shaking in room temperature. This was done for 10-15 minutes, three times.

4.3.2 Determination of protein concentration
Measurement of protein concentration was performed after concentrating the protein fractions from SEC. Nanodrop spectrophotometer wave-scan function was used to obtain an absorbance at 280 nm (where tyrosine and tryptophan absorb) using PBS as reference and an absorbance spectrum ranging from 450 to 250 nm. The obtained absorbance was used to calculate the protein concentration through Beer Lambert’s law using an extinction coefficient of 18450 M⁻¹cm⁻¹ for TTR monomer.

\[ A = C \times l \times \varepsilon \]

At times when the Nanodrop would give unreliable results, the spectrophotometer Hitachi 2001 was used to measure absorbance at 280 nm using 1 cm quartz cuvettes.

4.3.3 TTRwt fibrillogenesis in vitro
A very interesting feature of TTR fibrils is that the constructed fibrils in vitro are mostly structurally different to those seen in vivo. The following experimental attempts of fibrillation in vitro is to obtain the same TTR fibrils that are seen in vivo. The idea is to try different environmental conditions in terms of pH, salt concentration and through a posttranslational...
modification, such as a cleavage treatment to remove the N-terminal part of the monomer, using CNBr for both wild-type and T49M mutant.

4.3.3.1 Cleavage of unfolded TTR with CNBr
Truncations of a polypeptide chain at the N- or C-terminal is a rather common post translational modification in proteins. CNBr performs chemical cleavage of methionine at the C-terminal side in vitro [39], see Figure 9.

![Cyanogen bromide cleavage site](image)

**Figure 9.** A theoretical illustration of the obtained fragments after treating TTR-T49M with cyanogen bromide. The truncated fragment 50-127 is highly amyloidogenic, and in this project used in attempt to generate TTR fibrils morphological similar to those seen in TTR amyloidosis tissues. For the TTRwt only two fragments are theoretically obtained after being treated with CNBr, 1-13 and 14-127 since TTRwt has only one methionine in the sequence at position 13.

In the first modification using CNBr to TTRwt (concentration 143 µM), varying concentration of CNBr were tested. A CNBr molar excess of 97x and 6993x were used in the experiment on generating different TTR fibrils in vitro. However, for the TTR-T49M against TTRwt experiment, the CNBr concentration were lowered compared to the 6993x excess. Approximately 2 ml of TTRwt (concentration 94 µM) and TTR-T49M (concentration 72 µM) in 10 mM HCl buffer were treated with 126x and 163x molar excess of CNBr respectively by adding 100 µl of CNBr stock solution. The solutions were vortexed thoroughly and incubated at 37° C for 48 hours.

4.3.3.2 pH 2 treatment of unfolded TTR
For TTR fibrillogenesis in pH 2 buffer, TTR after the dialysis in 10 mM HCl was added to 10 mM HCl, 100 mM NaCl buffer reaching a final volume of 5 ml and a protein concentration of 0.2 mg/ml. To increase the fibrillation rate, it was incubated at 37° C for approximately 48 hours.

4.3.3.3 pH 4.4 treatment of native TTR
To reach pH 4.4, 4.5 ml of 55 mM acetic acid was mixed with 0.5 ml of TTR in PBS buffer reaching a final protein concentration of 0.2 mg/ml. pH was checked in control solutions. This was stored at 37° C for approximately 48 hours.

4.3.4 Fluorescence measurements with infinite M1000 PRO microplate reader
The Microplate reader infinite M1000 PRO (from Tecan) was used to measure the binding of several amyloid binding probes to native and fibrillated TTR using fluorescence absorbance and emission scan. The experiments consisted of a ligand titration triplicate of six amyloid binding probes to native TTR, six amyloid binding probes to TTRwt fibrils generated in varying conditions, fluorescence emission scans using seven amyloid binding probes along with an absorbance scan with CR to fibrillated TTRwt and TTR-T49M after treatment with CNBr.
The program software Magellan was used to run and analyse the data. The mode bottom-up was used in all measurements except for the absorbance scan with CR. For each type of experiment, an initial shaking of the plate was performed to mix the sample and remove air bubbles. The settings were as followed: 20 seconds of orbital shaking at 216 rpm, 3 mm amplitude. See Table 2 for wavelength settings.

Table 2. The wavelength settings for plate reader emission scan of seven amyloid binding probes used in the project.

<table>
<thead>
<tr>
<th>Probes</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-FTAA</td>
<td>450</td>
<td>480-700</td>
</tr>
<tr>
<td>hx-FTAA</td>
<td>450</td>
<td>480-700</td>
</tr>
<tr>
<td>h-FTAA</td>
<td>450</td>
<td>480-700</td>
</tr>
<tr>
<td>BTDSB</td>
<td>450</td>
<td>480-700</td>
</tr>
<tr>
<td>HS-169</td>
<td>530</td>
<td>560-800</td>
</tr>
<tr>
<td>HS-199</td>
<td>530</td>
<td>560-800</td>
</tr>
<tr>
<td>ThT</td>
<td>440</td>
<td>470-650</td>
</tr>
</tbody>
</table>

4.3.4.1 Titration triplicate experiment
The titration experiment was performed with 500 nM native TTRwt with an increase in probe concentration for every measurement, see Table 3. An emission bandwidth of 5 nm, excitation bandwidth of 10 nm, step size of 4 nm and a manual gain of 150 was used. Three replicates of the experiment were performed for each probe.

Table 3. Table illustrates wells in a 96-well assay plate. The preparation of one replicate is displayed in the table, total of three replicates were performed for each of six different amyloid probes.

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</tr>
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<tbody>
<tr>
<td>A</td>
<td>500nM TTR</td>
<td></td>
<td>0 nM probe</td>
<td>20 nM probe</td>
<td>50 nM probe</td>
<td>100 nM probe</td>
<td>250 nM probe</td>
<td>500 nM probe</td>
<td>750 nM probe</td>
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<tr>
<td>B</td>
<td>PBS</td>
<td>0 nM probe</td>
<td>20 nM probe</td>
<td>50 nM probe</td>
<td>100 nM probe</td>
<td>250 nM probe</td>
<td>500 nM probe</td>
<td>750 nM probe</td>
<td>1000 nM probe</td>
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4.3.4.2 Binding of amyloid probes to differently generated in vitro fibrils
Fibrillated TTRwt in three different conditions: CNBr treated TTR in 10 mM HCl, TTR in 10 mM HCl and 100 mM NaCl (pH 2), TTR in PBS and acetic acid (pH 4.4). A total of 600 μl sample from each stock of fibrillated TTR and pure buffer as reference was loaded into the wells of a 96-well plate with transparent bottom, 100 μl in each well. Six amyloid probes were added to the pre-filled wells to a final concentration of 300 nM, see Figure 10. The settings of the measurement were 100 gain, 4 nm step size, 5 emission and excitation bandwidth.
Figure 10. An illustration of how the experiment was set up in a “96-well assay plate”. The amyloid probes: p-FTAA, hx-FTAA, h-FTAA, BTDSB, HS-169 and HS-199 were separately added to all three types of TTR fibrils in their corresponding buffer with a final concentration of 300 nM. The Figure shows the general idea of how the preparation was performed, starting with filling the wells with buffer I, with and without fibrils and then adding ligand according to the image. This was repeated with all three buffers to measure the ligand & fibril effects in different fibril growth conditions.

4.3.4.3 Emission scan of amyloid probes bound to TTRwt fibrils and TTR-T49M fibrils after CNBr treatment

Reference buffer, truncated TTRwt (concentration 94 µM) and TTR-T49M (concentration 72 µM) were added to the wells, 100 µl in each well. 2 µl of seven amyloid binding probes from stock solutions was added separately to the wells, meaning one type of amyloid probe to a well consisting of reference buffer, truncated TTRwt and TTR-T49M. See Figure 11 for an illustration of how the loading of the plate was performed and the final ligand concentrations. The emission scan settings used were 150 gain, 1 and 4 nm step size, 5 emission and excitation bandwidth. See Table 2 for excitation wavelength and emission wavelength range.
Figure 11. An illustration of the set-up when loading samples to the “96-well assay plate” for the fluorescence emission scan of TTRwt and TTR-T49M using a plate reader. The amyloid binding probes used in this experiment: p-FTAA, hx-FTAA, h-FTAA, ThT, BTDSB, HS-169 and HS-199. The final concentration of the amyloid binding probes was 300 nM except for ThT which had a final concentration of 40 µM (133x excess in comparison to the other probes).

4.3.4.4 Absorbance scan of CR bound to TTRwt fibrils and TTR-T49M fibrils after CNBr treatment
Native TTRwt and TTR-T49M, truncated TTRwt and TTR-T49M after CNBr treatment was added to a 96-well assay plate, 100 µl in each well with a final concentration of 10 mM. PBS was added as a reference and the final concentration of CR in the wells was 5 µM, see Table 4. The settings used for the absorbance scan: step size 4 nm, absorbance scan range of 390-700 nm and 150 gain.

Table 4. Experimental set up of CR absorbance experiment with truncated wild-type and T49M mutant. One row of the 96-well assay plate A1-A10 was used and table shows what is present in each well.

<table>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>PBS</td>
<td>PBS + CR</td>
<td>Native wild-type TTR</td>
<td>Wild-type TTR fibrils</td>
<td>Native wild-type TTR + CR</td>
<td>Wild-type TTR fibrils + CR</td>
<td>Native T49M TTR</td>
<td>T49M TTR fibrils</td>
<td>Native T49M TTR + CR</td>
<td>T49M TTR fibrils + CR</td>
<td></td>
<td></td>
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4.4 Methods – Experiments of amyloid probes bound to transthyretin fibrils using fluorescence microscopy
4.4.1 Analysis of stained TTRwt fibrils with fluorescence microscopy
To analyse TTR fibrils in the fluorescence microscope, a preparation of microscope specimen was performed. From previous experiments using the plate reader for measuring amyloid probe binding to differently constructed TTR fibrils, the samples in the well containing TTR fibrils and amyloid binding probe and reference buffer with amyloid probe was transferred to microtubes. The microtubes containing TTR fibrils were centrifuged at 15000 RCF for 15 minutes to sediment the fibrils. A glass slide was put on top of a loading template (see Figure 12) and a reference buffer containing amyloid binding probe was loaded on a dot seen through the glass slide. TTR fibrils with amyloid binding probe was loaded onto the next dot.
When all samples had been loaded, the glass slide was left to dry. A droplet of fluorescence mounting medium from Dako was added to the glass slide and a cover glass was put on top to spread the mounting medium. The microscope specimens containing samples of TTR fibrils stained with amyloid binding probes were imaged using a fluorescence microscope (Leica DM6000B) equipped with a spectral cube (ASI). Same procedure was performed when preparing the specimen of TTR-T49M.

![Image](image.png)

*Figure 12. Loading template when preparing a specimen for studying amyloid probes bound to amyloid fibrils with the fluorescence microscope.*

### 4.4.2 Analysis of human carpal tunnel tissue stained with amyloid binding probes using fluorescence microscopy

Six paraffinized samples of thin carpal tunnel tissue sections of a Japanese patient with TTR amyloidosis on glass slides were provided from a previous study [40]. To proceed with staining of the tissue samples it was first deparaffinized by treating it in solutions in the following order: xylene for at least two hours and then rehydrated by incubation in 99 % ethanol, 96 % ethanol, 70 % ethanol, dH$_2$O and PBS for 10 minutes each [41]. The staining of the tissues was made by adding 200 μl of 0.5 μM amyloid binding probe, one glass slide of deparaffinized carpal tunnel tissue for each amyloid binding probe. The staining was performed for 30 minutes and was thereafter rinsed with PBS before adding one droplet of mounting medium from Dako. A cover glass was put on top of the glass slides and was left to try overnight.

The specimens were illuminated with light of specific wavelengths depending on the excitation wavelength of the amyloid probe used. Spectral images were taken with a camera linked from the fluorescent microscope to a computer and different region of interest (ROI) were selected on the image to display an emission spectrum of the selected region.

### 4.4.3 Quantification of TTR amyloid in carpal tunnel tissues stained with h-FTAA and hx-FTAA

The idea behind quantification of amyloid load in the carpal tunnel tissues in this project was to use 10x zoom and search for well characterized stained amyloid parts and image them. In the captured images, ROI markers were created for background, carpal tunnel tissue and TTR amyloid. These marked regions were selected and used as reference spectra, meaning that all pixels in the image are characterized as being most similar to one representative spectrum. A tool called “Spectral Unmixing” (SUN) was used to separate images into layers corresponding to the selected ROI’s similar fluorescence spectrum and intensity. Basically, one achieves a layer that corresponds to carpal tunnel tissue (with no amyloid) and one that corresponds to stained TTR amyloid using their equivalent reference spectra (selected ROI) which colours or marks the image depending on the pixel intensity. Additionally, a combined image of these two layers are given that is also used to quantify amyloid. When performing SUN, a threshold can be adjusted (by default 5). The threshold is a percentage of the maximum value (intensity at a specific wavelength) of the selected spectra in the library. All
data at wavelengths where the spectrum is lower than the threshold will be irrelevant and prevented from being analysed.

An image processing program called ImageJ is used to process the captured images taken from the fluorescence microscope that were applied with SUN. ImageJ is commonly used for scientific image analysis and processing. In this project the program is used to quantify amyloid in carpal tunnel tissue stained with hxFTAA and hFTAA. The function called “Colour Threshold” and a plugin called “Colour Pixel Counter” is utilized for amyloid quantification of the SUN images. Firstly, the background, referred to as the “black area” of the combined image is calculated in pixels and deducted from the total pixels of the image to get the area of tissue. Secondly, a calculation of the amyloid is performed using the SUN image corresponding to the stained amyloid. Colour pixel counter is used to select a colour of interest, additionally a threshold (30 by default) on pixel intensity for selected colour can be adjusted, this threshold determines what is referred as positive or negative. In this case, positive is referred to as amyloid. To decide the threshold, it is recommended to hover the mouse cursor over the image and by looking at the intensity displayed in the toolbar of ImageJ, decide what threshold is suitable. The plugin will then calculate the number of pixels considered as positive. Dividing the number of pixels representing amyloid with the total tissue pixels will give a percentage of amyloid in the tissue of the image. (In appendix, a point list with step by step instructions and screenshots of how ImageJ was used to quantify the spectral images can be found).
5 Results

5.1 Site-specific mutagenesis

Interestingly, there have been cases of truncated TTR fragments being abundant in cardiac amyloidosis. Ihse et al, mentions that there are a mix of full-length and C-terminal fragments starting at position 46, 49 and 52 found in most cases of TTR amyloidosis [42]. These C-terminal fragments are known to be highly amyloidogenic and have been a major amyloid factor in tissue biopsies connected with amyloid deposition in the heart. In order to study aggregation prone TTR fragments, two site specific mutations were performed, however, one of them being a backup experiment. The main objective was to achieve the synthetic mutation T49M to obtain one of the aggregation prone fragments through cyanogen bromide treatment. In case the result of T49M mutation would be unsuccessful another mutation S52P was made in parallel. This mutation is also known to yield an aggregation prone fragment through limited proteolysis under physiological conditions and fluid stirring [10].

After obtaining the PCR product of these two mutations, an agarose gel electrophoresis was performed which displayed presence of DNA, but whether the mutation was successful was hard to tell. To ensure that the mutation was successful, a transformation of the PCR products to competent E. Coli bacteria was made for sequencing. Unfortunately, a mistake of using electroporation with chemical competent E. Coli to save time, yielded in no colonies on the plates. The second transformation, however, went perfectly fine after transforming with the heat-shock method.

The second plasmid prep was sent for sequencing and showed that T49M mutagenesis had been successful but S52P unsuccessful. A previously prepared plasmid prep was stored in -20 °C and both T49M and S52P were sent for sequencing and both were successful.

5.2 Ligand titration of amyloid probes to native TTR

It has been suspected in previous experiments in the lab that the binding of amyloid probes to native TTR interfered with fluorescence during amyloid binding studies.

A titration experiment of amyloid probes to native tetrameric TTRwt was performed to study the binding and affinity. The amyloid probes p-FTAA and h-FTAA were known from previous experiments to bind native TTR. However, hx-FTAA, BTDSB, HS-169 and HS-199 had not been previously studied with TTR. A triplicate was performed to give a more accurate result, where the average intensity of amyloid probe and TTRwt were subtracted from the average intensity of amyloid probe in PBS (reference).

<table>
<thead>
<tr>
<th>Probes</th>
<th>K_d-value</th>
<th>Std. error</th>
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<tbody>
<tr>
<td>p-FTAA</td>
<td>703 nM</td>
<td>288 nM</td>
</tr>
<tr>
<td>hx-FTAA</td>
<td>177 nM</td>
<td>141 nM</td>
</tr>
<tr>
<td>h-FTAA</td>
<td>1549 nM</td>
<td>335 nM</td>
</tr>
<tr>
<td>BTDSB</td>
<td>666 nM</td>
<td>273 nM</td>
</tr>
<tr>
<td>HS-169</td>
<td>1411 nM</td>
<td>318 nM</td>
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<tr>
<td>HS-199</td>
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In Figure 13c, h-FTAA has the highest intensity difference between amyloid probe with native TTRwt and amyloid probe in PBS. This indicates that the presence of h-FTAA with
TTRwt has the most reactive interaction. p-FTAA, hx-FTAA and BTDSB were saturated at 2000 nM, whereas, h-FTAA and HS-169 required additional titrations to obtain saturation. From the $K_d$-values (Table 5), hx-FTAA appears to have the highest affinity for native TTRwt, followed by BTDSB, p-FTAA, HS-169 and h-FTAA. No binding occurred between HS-199 and native TTRwt, this is likely because HS-199 is a positively charged molecule.

Figure 13. Showing curve fitting with nonlinear regression using one site specific binding for saturation of amyloid binding probes to native TTRwt. The measurements were performed in triplicate and error bars are shown in blue. A) Saturation curve for p-FTAA with a $K_d$ value of 703 nM. B) Saturation curve for hx-FTAA with a $K_d$ value of 177 nM. C) Saturation curve
for h-FTAA with a Kd value of 1549 nM. D) Saturation curve for BTDSB with a Kd value of 666 nM. E) Saturation curve of HS-169 with a Kd value of 1411 nM. F) Standard plot of HS-199 showing no sign of binding. C & E had additional saturation measurements, up to 3000 nM for h-FTAA (C) and 5000 nM for HS-169 (E).

5.3 Analysis of in vitro generated TTRwt fibrils stained with amyloid binding probes using fluorescence microscopy

The results of the first in vitro fibrillation experiments of the project are shown below in graphs of emission spectra and corresponding spectral image (see Figures 14-22). TTR fibrils were stained with various of amyloid probes, including LCO’s in attempt to identify similar morphological structures to TTR fibrils found in tissues.

Figure 14. 20x zoomed image of p-FTAA stained TTRwt fibril generated in 10 mM HCl after being treated with entire CNBr process, including dialysis. Light of wavelength 436 nm was excited for p-FTAA staining and resulted in double peaks at 505 nm and 555 nm.

Figure 15. 20x zoomed image of p-FTAA stained TTRwt aggregates generated in pH 4.4 buffer. The image shows a different kind of TTR aggregate, this type was clumped together forming something that could be described as a gel-like aggregate. Double peaks of the emission spectra are not very obvious.
Figure 16. 20x zoomed image of hx-FTAA stained TTRwt aggregates generated in pH 4.4 buffer. Gel-like aggregate with no obvious defined double peaks in the emission spectra.

Figure 17. 20x zoomed image of h-FTAA stained TTRwt fibril generated in 10 mM HCl after treatment with CNBr. The double peaks are at the correct wavelengths (550 nm and 600 nm) if compared to previous studied TTR fibrils in tissues. However, the emission spectrum shape of both ROI’s of the fibril is significantly different to TTR fibrils in tissues.
Figure 18. 20x zoomed image of BTDSB stained to TTRwt aggregates generated in pH 4.4 buffer. Emission peak of BTDSB bound to amyloids are usually seen at 620 nm. In this image, BTDSB is bound to the cloud-like aggregate and excited with light of wavelength 480 nm and an emission peak is seen at approximately 620 nm.

Figure 19. 20x zoomed image of HS-169 stained TTRwt fibrils generated in pH 2 buffer. Emission peak of M1 at approximately 700 nm. M0 seem to be more of a background.
Figure 20. 20x zoomed image of HS-169 stained TTRwt fibrils generated in pH 4.4 buffer. Emission peaks seen at approximately 680 nm.

Figure 21. HS-199 stained TTRwt fibrils generated in pH 2 buffer. M0 emission peak seen at 670 nm and M1 emission peak at 680 nm.

Figure 22. HS-199 TTRwt aggregates generated in pH 4.4 buffer. Emission peaks seen at approximately 670 nm.
5.4 Experimental procedures and analysis with TTR-T49M and TTRwt
Cleavage of TTR-T49M with CNBr was performed to obtain a truncated TTR fragment (50-127) prone to self-aggregate and form the TTR amyloid fibrils associated with TTR amyloidosis. Cleavage of TTRwt was performed in parallel, as a reference for comparisons between the two forms. The fibrils were stained with amyloid binding probes and experimental analyses were performed with fluorescence spectroscopy and fluorescence microscopy to obtain emission spectra and spectral images of fibrils in interaction with amyloid binding probes.

5.4.1 Electron microscopy images of TTRwt and TTR-T49M after CNBr treatment
The loading of my samples to a TEM grid was performed by a post doc (Ganesh Mohite) who also prepared samples for electron microscopy imaging and the electron microscopy images were taken by my examiner. The images show fine structured amyloid fibrils.

![TEM images](image)

**Figure 23.** Electron microscopy images of TTRwt and TTR-T49M fibrils after CNBr treatment. TEM grids were loaded with 10x diluted TTR fibrils after approximately 48 hours incubation in 37°C. A1-3, images of TTR-T49M fibrils with magnification of 100k. B1-B3, images of TTRwt fibrils with magnification 25k, 60k and 100k respectively.

5.4.2 Cleavage of TTRwt and TTR-T49M with CNBr
SDS-PAGE was run on native, unfolded and after CNBr cleavage of both TTR-T49M and TTRwt. This was performed to confirm the presence of fragment 50-127 in the gel and if the cleavage was successful. In Figure 24, in well 5 and 10, changes of TTR has occurred after being modified by over 100-fold excess of CNBr. TTR aggregation has occurred for both forms, however, there are differences. TTRwt seem to have a more smeared aggregation pattern with aggregates stuck at the well and trails of aggregates along the travel direction. The aggregation pattern of the mutant TTR is less smeared and appears to have a band-like pattern, indicating intermediate forms of aggregates. The dye markings below 15 kDa highlighted by red circles could imply smaller fragments of TTR, perhaps the target fragment 50-127.
Figure 24. SDS-PAGE of resulting CNBr treatment of TTRwt and TTR-T49M. Well 1: stained protein ladder from Bio-Rad. In well 5, TTRwt after being chemically modified by 125x excess of CNBr for 2 weeks, clear traces of aggregates are seen by the long trail of dye resulting in a less intensive band at ~ 15kDa, indicating a lower concentration of monomeric TTR. Well 10 showing treatment of 163x excess of CNBr with TTR-T49M for two weeks. A zoomed in protein ladder is shown for reference.

5.4.3 Interactions of amyloid binding probes with TTR-T49M and TTRwt fibrils after CNBr treatment using absorbance and fluorescence spectroscopy

For this experiment it was known from previous studies that p-FTAA, h-FTAA, ThT and CR were binding TTR fibrils. ThT and CR were references to confirm amyloid fibril presence. The two LCO’s p-FTAA and h-FTAA would confirm whether the in vitro generated fibrils were structurally similar to the amyloid fibrils present in TTR amyloidosis tissues. The remaining amyloid binding probes, BTDSB, HS-169, HS-199 and hx-FTAA and their interactions with TTR amyloid fibrils were to be studied. Hx-FTAA having the same feature as p-FTAA and h-FTAA in characterizing fibril structure.
Figure 25. Absorbance spectrum of CR to native and CNBr treated TTRwt (left) and TTR-T49M (right). Image below the graphs: TTR concentration was 10 μM and CR concentration 5 μM. A) Native TTR-T49M with CR. B) CNBr treated TTR-T49M with CR. C) CNBr treated TTRwt with CR. D) Native TTRwt with CR. In B, we see clearly that the aggregates have bound to all CR molecules, thus the red pellet and clear solution. In comparison to the other three, A, C and D, the solution remain red and no sight of any red pellet.

The result from the CR binding experiment to TTR-T49M and TTRwt aggregates seen in Figure 25, showed that TTR-T49M after CNBr treatment had a greater yield of TTR aggregates. This can be seen in microtube B, where a red pellet has been formed after adding CR, and in the (right) absorbance spectra by looking at the wavelength shift towards the red spectrum [43].

Graphs below show the emission spectra of amyloid binding probes in interaction with TTR-T49M and TTRwt fibrils formed by CNBr cleavage, along with amyloid binding probes in PBS as reference in the plate reader (see Figures 26-32). Note that the concentration varied between WT and T49M TTR, 94 μM and 72 μM respectively. The concentration of probes were 300 nM except for ThT that had 40 μM.
Figure 26. A1 emission spectrum of p-FTAA with (green) and without (black) TTRwt fibrils after CNBr treatment. First peak at 508 nm and second at 545 nm. A2 normalized graph of A1 showing slight blue shift after p-FTAA binding with TTR fibrils. B1 emission spectrum of p-FTAA to T49M-TTR fibrils (green) and as a reference (black). Approximately the same intensity as the reference and no double peaks shown, but a slight blue shift. B2 normalized graph of B1.

Figure 27. A1 emission spectra of hx-FTAA in PBS (black) as a reference and with TTRwt fibrils (green). First peak at 525 nm and second 555 nm, blue shifted. A2 normalized graph of A1. B1 emission spectra of hx-FTAA with (green) and without (black) TTR-T49M fibrils, peaks are located at the same wavelengths as the WT and spectrum is blue shifted. B2 normalized graph of B1. In B1 we see a higher intensity despite the lower protein concentration compared to A1. The WT appears to be a little more blue shifted by comparing A2 against B2.
Figure 28. A1, emission spectra of h-FTAA with TTRwt fibrils (green) and as reference in PBS absent of fibrils (black). No double peaks, indicating different forms of protein aggregates and not those seen in TTR amyloid tissues. A2, normalized graph of A1, a slight blue shift is seen. B1 shows emission spectra of hx-FTAA with T49M TTR fibrils and B2 normalized graph of B1. In B1 we see a higher intensity compared to A1.

Figure 29. A1, emission spectra of BTDSB with (red) and without (black) TTRwt fibrils. A2, normalized graph of A1, showing a red shift when bound to TTR fibrils. B1, emission spectra of BTDSB with (red) and without (black) T49M TTR fibrils. B2, normalized graph of B1, showing red shift.
Figure 30. A1, emission spectra of ThT with (blue) and without (black) WT TTR fibrils. A2, normalize graph of A1. B1, emission spectra of ThT with (blue) and without (black) T49M TTR fibrils. B2, normalized graph of B1. Both types look similar with a slight blue shift upon binding TTR amyloid fibrils.

Figure 31. A1, emission spectra of HS-169 with (red) and without (black) TTRwt fibrils. A2, normalized graph of A1. B1, emission spectra of HS-169 with (red) and without (black) T49M TTR fibrils. B2, normalized graph of B1.
The fact that the characterising double peaks are not seen in most of the LCO’s stained recombinant TTR amyloid, indicates that the samples had a very low amount of TTR amyloid fibrils but could also be due to interference from other types of TTR formed aggregates and native TTR present in the samples. Low concentration of actual TTR fibrils, higher concentration of other types of aggregates and interference from native TTR, which also binds amyloid probes is most likely the reason to the different emission spectrum observed. Fluorescence microscopy takes the analysis and determination of amyloid fibril to a next level, since there is no need to blindly rely on the emission spectra from the fluorescence plate reader measurements. Fluorescence microscopy, in contrast to in solution spectroscopy, allows for specific analysis of fibrillar objects rather than on the average fluorescence from the entire sample.

5.4.4 Spectral imaging of amyloid binding probes with TTR-T49M and TTRwt fibrils after CNBr treatment using fluorescence microscopy

In this experiment, the generated recombinant TTR fibrils of TTRwt and TTR-T49M after CNBr treatment were studied using fluorescence microscopy. This experiment strives for finding in vivo similar structured fibrils among the generated recombinant TTR fibrils. Using LCO’s makes this search possible by comparing the recombinant TTR fibril spectrum with an actual in vivo ATTR spectrum (see Figure 43). Specifically, by comparing the emission spectrum double peaks, fluorescence intensity and wavelength shifts. For the non-LCO amyloid probes, obtained emission peaks at selected ROI notifies on amyloid positive, which means determination of amyloid type is not possible using only the emission spectrum.

Figures below show the spectral images of various amyloid binding probes bound to TTR amyloid taken with fluorescence microscopy. Additionally, for each spectral image, normalized emission spectra and the maximum fluorescence intensity of the corresponding emission spectrum from selected ROI’s are shown in a bar chart. Arrows points at the
selected ROI’s (seen as squares in the image), and colours indicates which ROI belonging to which emission spectrum and bar in the bar chart (see Figures 33-38).

Figure 33. Showing a 10x zoomed spectral image of (CNBr treated) TTR-T49M stained with p-FTAA. The double peaks are located at approximately 505 nm and 555 nm.

Figure 34. Showing a 10x zoomed spectral image of (CNBr treated) TTRwt stained with hx-FTAA. M0 appears to be the only aggregate with double peaks resembling TTR amyloid. First peak at 520 nm and second at 570 nm.
Figure 35. Showing a 10x zoomed spectral image of (CNBr treated) TTR-T49M stained with hx-FTAA. Assumed TTR fibrils (M0-M2) are embedded in a gel-like TTR aggregate. However, M0 showing most resemblance of TTR fibril in terms of double peaks and intensity in the emission spectra.

Figure 36. Showing a 20x zoomed spectral image of (CNBr treated) TTR-T49M stained with h-FTAA. In the image, the assumed TTR fibrils (M0-M2) are embedded in a gel-like TTR aggregate (M3). Clear differences in emission spectrum shape can be seen, where M3 is more blue shifted, does not have the double peaks and has a significantly lower fluorescence intensity.
Figure 37. Showing a 10x zoomed spectral image of (CNBr treated) TTRwt stained with h-FTAA. All selected ROI’s of the spectral image except for the background, displays double peaks at ~550 nm and 600 nm in the emission spectra, indicating TTR amyloid fibrils.

Figure 38. Showing a 10x zoomed spectral image of (CNBr treated) TTR-T49M, observed as a gel-like aggregate. Stained with ThT.

In the case of h-FTAA (Figure 36 & 37) stained to TTRwt and TTR-T49M, the characteristic double peaks are located at approximately 550 nm and 600 nm which coincides to previous studies related to TTR amyloidosis [4,5], implying that generated recombinant TTR are reasonably similar to the *in vivo* ATTR. In many cases, especially seen in Figure 34, 35 and 36, a gel-like TTR aggregate is seen close to the fibrillar LCO positive aggregates. This gel-like TTR aggregate appeared to be more present in TTR-T49M fibrillations in comparison to TTRwt. The gel-like aggregate was ThT positive when stained with 40 μM ThT, see Figure 38.
5.5 Quantification of TTR amyloid load in carpal tunnel tissue

Quantification of amyloid in two carpal tunnel sections using the probes hx-FTAA and h-FTAA separately for each section was performed. Eight images of various locations in the carpal tunnel was captured for each probe. Two images with highest (Figure 39 & 41) and lowest (Figure 40 & 42) amyloid content from each probe will be presented in the results, the rest of the images and the corresponding quantification results can be found in appendix. Note that only the pixels corresponding to tissue is used for quantification, the black background is not included.

![Figure 39. Showing the spectral images included in the quantification and a bar chart of resulting amyloid content. Image of h-FTAA bound to ATTR with highest amyloid percentage. A) Original image of a small fraction of one of the carpal tunnel sections used. Selected ROI from the original image were used to obtain the SUN images. B) Spectral un-mixed image of the tissue, selected ROI corresponding to tissue is used as reference spectrum for the image. C) Spectral un-mixed image of amyloid in the tissue, selected ROI corresponding to amyloid is used as reference spectrum for the image. D) A combination of B and C. C & D was used for quantification of amyloid content with ImageJ.](image-url)
Figure 40. Showing the spectral images included in the quantification and a bar chart of resulting amyloid content. Image of h-FTAA bound to ATTR with lowest amyloid percentage. A) Original image of a small fraction of one of the carpal tunnel sections used. Selected ROI from the original image were used to obtain the SUN images. B) Spectral un-mixed image of the tissue, selected ROI corresponding to tissue is used as reference spectrum for the image. C) Spectral un-mixed image of amyloid in the tissue, selected ROI corresponding to amyloid is used as reference spectrum for the image. D) A combination of B and C. C was used for calculation of amyloid content and D for background calculation with ImageJ.
Figure 41. Showing the spectral images included in the quantification and a bar chart of resulting amyloid content. Image of hx-FTAA bound to ATTR with highest amyloid content. A) Original image of a small fraction of one of the carpal tunnel sections used. Selected ROI from the original image were used to obtain the SUN images. B) Spectral un-mixed image of the tissue, selected ROI corresponding to tissue is used as reference spectrum for the image. C) Spectral un-mixed image of amyloid in the tissue, selected ROI corresponding to amyloid is used as reference spectrum for the image. D) A combination of B and C. C & D was used for quantification of amyloid content with ImageJ.
5.6 Comparisons of ex vivo amyloid and in vitro generated amyloid

Emission spectra from in vitro recombinant TTR fibrils stained with hx-FTAA and h-FTAA after CNBr treatment was compared with the emission spectra from amyloid in carpal tunnel, see Figure 43. ATTR-T49M and ATTR from tissues stained with hx-FTAA (left emission spectra) shows most similarities in their spectrum, whilst the ATTRwt does not have the same emission spectra shape. As for ATTR stained with h-FTAA, ATTRwt and ATTR-T49M have similar emission spectra and double peaks (550 nm and 600 nm), while ATTR in tissue is more blue shifted and with not as characterized double peaks. Seen from previous studies of h-FTAA bound to ATTR in fat tissue [4], the double peaks also appeared at 550 nm and 600 nm.
Figure 43. Showing the comparisons made between in vitro recombinant generated TTR fibrils against ex vivo ATTR (carpal tunnel tissue). Left: emission spectra and their corresponding spectral images of hx-FTAA stained to ATTR. Right: emission spectra and spectral images of h-FTAA stained to ATTR. The spectral images and emission spectra with maximum fluorescence intensity of the TTR amyloid carpal tunnel tissues are found in appendix.

6 Discussion

6.1 Site-specific mutagenesis
The first plasmid prep of the mutagenesis products TTR-T49M and TTR-S52P were both successful. However, two plasmid preps were performed, the second round was sent for sequencing first and ended up with one being successful. Then the plasmids from the first round was sent and both ended up successfully. The reason to the unfortunate outcome of one of the plasmids was undoubtedly due to something faulty occurring between the transformation and cultivation steps. The cultivation of the first round was over a few hours while the second round was incubated overnight.

6.2 Ligand titration of amyloid binding probes to native TTR
From the triplicate titration experiment of ligands to native TTR the important information of whether the amyloid binding probes would bind native TTR was determined. This information is significant for the fibrillation experiments because all native TTR may not turn into fibrils and could be present as a mixture with the generated TTR fibrils. This could affect the binding and signalling of the probes to fibrillated TTR. In this experiment 5 out of 6 amyloid binding probes were binding to native TTR, meaning that native TTR could undoubtedly affect the measurements of the fibrillation experiments. This experiment was initially planned for studying the binding of amyloid probe to native TTR. The thought of native TTR remaining in the fibril experiments and thus could affect the results of the measurements between amyloid probe to TTR did not appear in my mind during the experimental procedures. However, one attempt on separating native TTR from fibrillated TTR was tested but the outcome was a failure, most likely due to the TTR being stuck on the filter used, since no signal after the separation attempt was observed on the fluorescence plate reader measurements. Not more than one attempt on the separation was performed due to this factor appearing late in the project process, no time could be spared, and other experiments had to be prioritized.
6.3 Cleavage of TTR-T49M and TTRwt with CNBr
The idea of using CNBr to fragment TTR by cleaving methionine at the C-terminal side proved to be efficient theoretically, because methionine is a rare amino acid in the TTR sequence, and a mutation of a methionine is easily implemented to obtain one of the TTR amyloidotic prone fragments, see Figure 9. Treating TTR-T49M with CNBr did show a considerably increase in protein aggregation, especially when compared to CNBr treated TTRwt. The difference was specifically seen in the test tubes, where the solution was turbid, and a pellet formed for TTR-T49M while TTRwt was a lot clearer.

In the SDS-gels, traces of the fragment 50-127 aa could not be seen, except for one where it could possibly indicate traces of this fragment, Figure 24. Apart from the cleavage of methionine, there are suspicions of other modifications occurring caused by CNBr. The majority of the formed TTR aggregates using CNBr was not the target fibrils found in ATTR tissues, the other TTR aggregate variant was morphologically different in the microscope and had a different emission spectrum.

6.4 Generation of in vitro TTRwt fibrils in different conditions
The formation of in vivo-like ATTR using in vitro methods was more difficult than anticipated. Results from the measurement of emission spectra using fluorescence microscopy with LCO stained to recombinant ATTR showed some predicted double peaks. However, most LCO’s stained recombinant fibrils did not have any obvious similarities. The fact that it was difficult to find amyloid when looking at the microscopy specimen using fluorescence microscopy hints that something happened with the samples during the addition of fluorescence mounting medium (Dako).

6.5 Experimental procedures with TTR-T49M and TTRwt after CNBr treatment
The second attempt on generating in vivo-like ATTR with TTR-T49M was considered more successful, including the TTRwt that was performed in parallel and in same conditions. It was relieving to observe differences of these two variants during the whole experimental procedure, from generation of recombinant ATTR-like fibrils to experimental measurements using CR absorbance, electron microscopy and fluorescence microscopy.

In the comparison section (5.6) there are noticeable similarities between the ATTR from tissues and the recombinant generated TTR stained with hx-FTAA and h-FTAA. The electron microscopy images show recognizable amyloid-like fibril structures for both TTRwt and TTR-T49M (Figure 23), however, the yield of these fine amyloid-like fibrils was low, and the majority of the recombinant amyloid consisted of the gel-like aggregate which did not stain with LCO’s.

6.6 Quantification of ATTR in carpal tunnel tissues
ImageJ is well suited for image quantification purposes and could be used to quantify most fluorescence microscopy images. For the study in this project, it was convenient to use the plugin “Colour Pixel Counter” due to only one colour had to be considered. The important factor in obtaining accurate quantification results is dependent on the resolution and contrast of the images. Most images of hx-FTAA stained carpal tunnel tissues were quite hard to distinguish the tissue (blue colour) from background (black colour). This was generally due to the amyloid (white/yellow) having a much higher intensity compared to the tissue, see Figure 41 & 42. The images of h-FTAA stained carpal tunnel tissues were easier to
distinguish and made it easier to compare whether the quantification result was fairly agreeable. Overall, in this project a useable method for quantification of amyloid in patient tissue specimen is presented.

6.7 Future prospects
For future related studies to this project with TTR, it would be important to follow up whether the target fragment 50-127 was acquired or if the CNBr modification changed the monomeric TTR in a different way. This could be studied with mass spectrometry. Certainly, there are other ways of obtaining aggregation prone TTR fragments through various of proteolytic reactions, for example the backup mutant S52P could be cleaved with chymotrypsin [10]. Studying other forms of aggregation prone fragments would be interesting for comparisons to the results from this project and contribute with new knowledge for TTR research.

It would be essential to separate native TTR from the generated recombinant TTR amyloid fibrils before performing fluorescence measurements to minimize any interferences that may occur to the amyloid probes. Designing a protocol for separation of native TTR to TTR fibrils with a minimal protein loss and measuring the separated samples for validation would be very beneficial.

6.8 Ethical and societal considerations
All hazardous chemicals were handled with care and safety precautions. The human ATTR carpal tunnel tissues provided for this project was used strictly for researching purposes used in previous studies.

Currently, there are several treatments against TTR amyloidosis. However, the major issue lies in finding the individuals with or at risk of developing TTR amyloidosis. The fact that TTR amyloidosis is a systemic disease and is commonly misdiagnosed and difficult to distinguish from diseases affecting similar organs with similar symptoms, the most reliable way of diagnosing TTR amyloidosis is through tissue biopsies using amyloid binding dyes. Many patients with TTR amyloidosis are diagnosed late and the amount of deposited TTR amyloid in tissues does impact the efficiency of the treatments, minimizing the survival rate. From research studies, there are linkage of carpal tunnel syndrome bearers being at risk of developing TTR cardiac amyloidosis. Since the on-set age of SSA is around 65 years and onwards, older patients suffering from carpal tunnel syndrome could be checked for TTR cardiac amyloidosis for potential early treatment.

This project consists of studies on potential novel amyloid binding probes that could function as a clinical diagnosis tool for TTR amyloidosis. Hopefully, will the obtained information contribute to related studies on TTR amyloidosis.

7 Conclusions
Out of six novel amyloid binding probes used in this project, five being anionic and were binding to native TTR with possibility of interfering with amyloid detection. HS-199 did not bind to native TTR. hx-FTAA had the best Kd-value (177 nM) out of the three LCO’s used in the titration experiment.

The yield of in vivo-like ATTR fibrils was low for both TTR-T49M and TTRwt after CNBr treatment. This is the first time it has been shown that TTR fragment 14-127 appears
amyloidogenic. The majority of produced TTR aggregates through this method was observed as a gel-like aggregate with no double peaks in the emission spectrum from LCO’s but showed some ThT affinity at high concentration. In the comparison between recombinant TTR fibrils and ATTR in carpal tunnel tissues, the emission spectra of these variants had similar characteristics, followed by electron microscopy imaging, fine ATTR fibrils of TTR-T49M and TTRwt was observed. From the CR experiment, all CR molecules had bound with generated TTR-T49M aggregates which was seen in the microtube (Figure 25). From the absorbance spectra of TTR-T49M and TTRwt both were more red shifted, which occurs when CR binds amyloid.

A method to quantify amyloid in tissue specimen was developed in this project. Quantification of ATTR carpal tunnel tissues could be improved by not using an image with too high amyloid intensity versus tissue, as in the case for the hypersensitive hx-FTAA. This could be solved by decreasing the staining concentration of amyloid probe and though adjustments in the fluorescence microscopy settings.

The obtained results will hopefully be of help for further studies related to generation of in vivo-like ATTR using in vitro methods, quantification of amyloid tissues and amyloid probe interactions with both native TTR and ATTR fibrils. The novel amyloid binding probes will hopefully be of use for improved diagnosis of TTR amyloidosis in biopsy samples early in the disease course and reduce misdiagnosis.
References


Appendix

Primers used for mutagenesis

**TTRT49M**
Forward: 5’ CTCTGGGAAAAATGAGTCTGGAGAGC 3’  
Reverse: 5’ GCAAATGGCTCCCAGGTG 3’

**TTR52S**
Forward: 5’ AACCAGTGAGCCTGGAGAGCTGC 3’  
Reverse: 5’ TTCCCAGGCAAATGGC 3’

Raw data and spectral images of carpal tunnel tissues stained with h-FTAA and hx-FTAA

In this section you find the spectral un-mixed images of amyloid (yellow) and the combined image of amyloid and tissue of h-FTAA and hx-FTAA stained carpal tunnel tissues including their emission spectra and fluorescence intensity maximum. Tables with the threshold used, raw data and results obtained using ImageJ for the quantification can also be found below.

**Table 6. Raw data from quantification of h-FTAA stained carpal tunnel tissues.**

<table>
<thead>
<tr>
<th>(Pixels)</th>
<th>Total image</th>
<th>Background</th>
<th>Amyloid</th>
<th>Tissue</th>
<th>Tissue with no amyloid</th>
<th>% Amyloid in tissue</th>
<th>ImageJ Threshold (1-255)</th>
<th>Threshold Colour Pixel Counter</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-FTAA A</td>
<td>625616</td>
<td>87609</td>
<td>330373</td>
<td>538007</td>
<td>207634</td>
<td>61.4%</td>
<td>15</td>
<td>40</td>
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<tr>
<td>h-FTAA B</td>
<td>625616</td>
<td>27934</td>
<td>190483</td>
<td>597682</td>
<td>407199</td>
<td>31.9%</td>
<td>35</td>
<td>65</td>
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<tr>
<td>h-FTAA C</td>
<td>625616</td>
<td>106332</td>
<td>124125</td>
<td>519284</td>
<td>395159</td>
<td>23.9%</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>h-FTAA D</td>
<td>625616</td>
<td>47263</td>
<td>182222</td>
<td>578353</td>
<td>396131</td>
<td>31.5%</td>
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<td>75</td>
</tr>
<tr>
<td>h-FTAA E</td>
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<td>209075</td>
<td>145468</td>
<td>416541</td>
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<td>75</td>
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<tr>
<td>h-FTAA F</td>
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<td>61523</td>
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<td>564093</td>
<td>278102</td>
<td>50.7%</td>
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<tr>
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<td>345863</td>
<td>119084</td>
<td>65.6%</td>
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<td>35</td>
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Table 7. Raw data from quantification of hx-FTAA stained carpal tunnel tissues.

<table>
<thead>
<tr>
<th>(Pixels)</th>
<th>Total image</th>
<th>Background</th>
<th>Amyloid</th>
<th>Tissue</th>
<th>Tissue with no amyloid</th>
<th>% Amyloid in tissue</th>
<th>ImageJ Threshold (1-255)</th>
<th>Threshold Colour Pixel Counter</th>
</tr>
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<tbody>
<tr>
<td>hx-FTAA A</td>
<td>625616</td>
<td>247637</td>
<td>121622</td>
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<td>hx-FTAA B</td>
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<td>125367</td>
<td>169255</td>
<td>500249</td>
<td>330994</td>
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<td>65</td>
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<tr>
<td>hx-FTAA C</td>
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<td>172427</td>
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<td>329275</td>
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<td>90</td>
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<td>186413</td>
<td>423551</td>
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<td>75</td>
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<tr>
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<td>264702</td>
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<td>20</td>
<td>75</td>
</tr>
<tr>
<td>h-FTAA G</td>
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<td>467846</td>
<td>241887</td>
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How the quantification was performed using ImageJ
Step by step of how the quantification was performed using ImageJ.

1. Calculate the background of the image by clicking on Image, Adjust and Colour Threshold.
2. In brightness, drag both the upper and lower bar to zero. Start adjusting the lower bar to mark the background (black area) of the image, seen as red in the image below.

3. Press on the select button to select the marked area.
4. Calculate the selected area by clicking on Analyse and then measure or Ctrl + M.
5. A result tab with calculated area in pixels is displayed.
6. To calculate the amyloid content of the spectral unmixed amyloid image, download the plugin Colour Pixel Counter.  
7. Before calculating the amyloid content, a threshold for Colour Pixel Counter must be decided and a colour selected. This can be done by clicking on the image and using the mouse cursor to hover around areas of the image to consider a suitable threshold, the value of the threshold and above will be considered as amyloid.  

8. Click on Plugins and select Colour Pixel Counter. Select colour, uncheck Display particle size and enter a threshold and press OK. The amount of pixels referred as amyloid, total pixels of the image and a percentage of number of amyloid pixels versus the total number of pixels are displayed.