PROJECT FINAL REPORT

Publishable summary report

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Executive summary

The objective of LUPAS was to develop novel molecular agents and methods for use within diagnostic imaging of Alzheimer's disease and prion diseases. Our concept relied on reporter molecules based on luminescent conjugated polymers, LCPs. The LCP molecules target pathogenic protein aggregates associated with these diseases and LCPs target these lesions with high selectivity and specificity. The objective was also to investigate potential therapeutic potency of the LCP imaging agents.

The work was organized in several interdependent layers of experimentation:

- Synthesis of LCPs and nanoparticles
- Synthesis of pure amyloidotic molecular targets
- Assay development – Fluorescence spectroscopy and Magnetic resonance imaging
- Development of disease models
- Assay of LCPs in tissue sections
- In vivo imaging – 2P fluorescence and Magnetic resonance imaging
- Therapeutic potential in prion model systems
- Therapeutic potential in vivo in Alzheimer disease models

LUPAS was focused on providing new knowledge in chemical design and smart targeting of misfolded protein amyloid lesions in Alzheimer´s disease and prion diseases. The LUPAS partners were assembled from a wide range of areas ranging from experts within organic synthetic chemistry, synthetic nano-chemistry, amyloid structure, prion disease' Alzheimer's disease, magnetic resonance imaging, multi-photon physics and hyper spectral imaging. This group formed the critical mass of competences needed to reach the project's ambitious goals.

The main project outcomes affords visualization of dynamics and biochemical activity of pathological processes of Alzheimer's disease and prion diseases in real-time from the molecular level to the organ and full body scale. Most importantly, novel molecular insight regarding the pathological hallmarks of these diseases and the specific chemical requirements for optimal amyloid ligands was concluded.

Dissemination activities included scientific article publications, conference presentations, amyloid workshop for the pathology community and sample shipments to two dozens of labs worldwide.

Summary description of project context and objectives

LUPAS

Innovative diagnostics and therapy for neurodegenerative diseases

Funded by the European Union under the 7th Framework Programme

Project context

The European population is aging. As a consequence age-related neurodegenerative diseases such as Alzheimer's disease are on a steady rise reaching epidemic proportions of serious concern to the afflicted patients and their relatives. These horrific diseases put a heavy burden on society including a decade of heavy dependence on caregivers culminating with care at nursing homes. If this epidemic is not sequestrated it is projected to threaten the core of the well-fare societies of all European Nations. The culprit within these diseases is accumulated misfolded protein molecules, called amyloids, which corrupt normal cellular functions and cause disease. Amyloids are self-perpetuating fibrillar states, which can hide for decades within affected individuals and replicate with immense efficiency, especially in the elderly. We, as a community, still stand short in delivering definite diagnosis or benign disease-modifying treatment or cure to any neurodegenerative disease. The only way to fight these diseases is long-term focused high quality research.

Within the LUPAS project we developed novel molecular agents and methods for use within diagnostic imaging that relies on reporter molecules based on luminescent conjugated polymers, LCPs. The LCP molecules target the pathogenic protein aggregates with high selectivity and specificity. Candidate lead molecules have also been shown to have therapeutic potential.

Consortium and work plan

The LUPAS partners were assembled from a wide range of areas ranging from experts within organic synthetic chemistry, synthetic nano-chemistry, amyloid structure, prion disease, Alzheimer's disease, magnetic resonance imaging, multi-photon physics and hyper spectral imaging. This group formed the critical mass of competences needed to reach the project's ambitious goals.

The partner organizations in the LUPAS consortium are: Linköping University (Sweden), Université Claude Bernard Lyon 1 (France), University of Tübingen (Germany), Norwegian University of Science and Technology (Norway), Zürich University Hospital (Switzerland),

Charité Universitätsmedizin Berlin (Germany), Applied Spectral Imaging (Israel) and Genovis AB (Sweden).

Most importantly the project was organized according to seven work packages that defined the work plan (Figure 1).

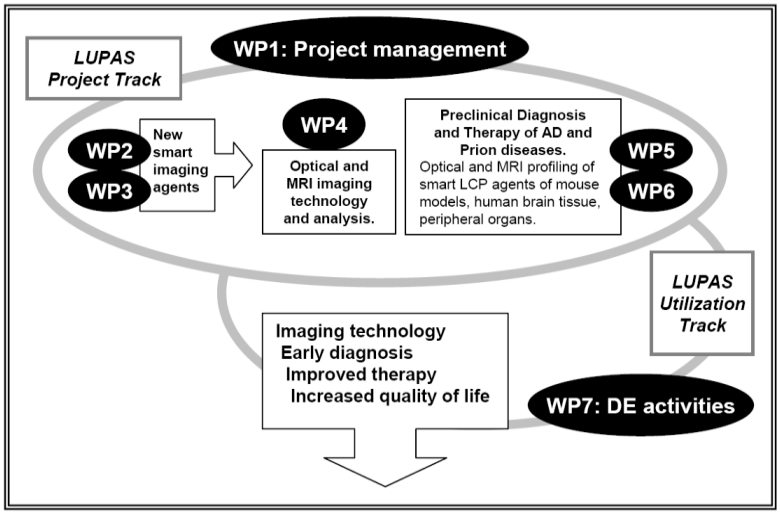


Figure 1: *The LUPAS program tracks for development of novel LCP based methods.*

The LUPAS project was described by five technical work packages aimed at developing novel optical and MRI imaging agents, towards the objectives of proving smart tools for biomedical research, for prediction, diagnosis, monitoring and prognosis of diseases, and for support and guidance of therapeutic interventions within the field of chronic neurodegenerative diseases.

- Work package 1 entailed the management of the consortium.
- Work package 2 generated LCP molecular reagents, reaction protocols and defined amyloidotic molecular targets (AMTs) for implementation in WP 5, WP6 and for grafting onto a MNP for MRI in WP 3.
- Work package 3 generated novel imaging agents for magnetic resonance imaging (MRI).
- Work package 4 constituted an arena for advanced optical spectroscopic characterization and nuclear magnetic resonance (NMR) imaging of LCP or LCP-MNP stained material from WPs 2-3 and WPs 5-6.
- Work package 5 was dedicated to investigation of AD transgenic mouse and human AD material for LCP and LCP-MNP diagnosis, amyloid biomarkers, and experimental therapeutic intervention.
- Work package 6 was dedicated to investigation of prion diseases from mouse and human material for LCP and LCP-MNP diagnosis, amyloid biomarkers, and experimental therapeutic intervention.

- Work package 7 was dedicated to the execution of dissemination and exploitation activities of project results. Specifically, the LUPAS project will make available the new smart imaging agents for clinical use with external non-profit stake-holders such as University hospitals and related research groups.

Results – in brief

LUPAS was a 3 year focused research project under the 7th Framework Programme. Significant achievements were reached which is well reflected by a surge of interest from the research community to use the LCP technology. Other indications of success are the high citation rate of publications from the LUPAS research groups. By combining the diversity of scientific knowledge available within the consortium, progress towards the development of multimodal amyloid ligands for non-invasive imaging was achieved. The outcome affords visualization of dynamics and biochemical activity of pathological processes of Alzheimer's disease and prion diseases in real-time from the molecular level to the organ and full body scale. Most importantly, novel molecular insight regarding the pathological hallmarks of these diseases and the specific chemical requirements for optimal amyloid ligands was concluded.

One of the major tasks within LUPAS was to develop novel luminescent conjugated polythiophenes (LCPs) that can be utilized as tools for selective identification of protein deposits, the pathological hallmarks of Alzheimer's disease and prion diseases.

LCPs are fascinating molecules that behave like structural chameleons. When an LCP binds to its amyloid target, the LCP changes shape. The LCP thereby also alters its color depending on the amyloid structure (Figure 1). Hence it reports different outcome signals which can be

detected by a range of techniques.

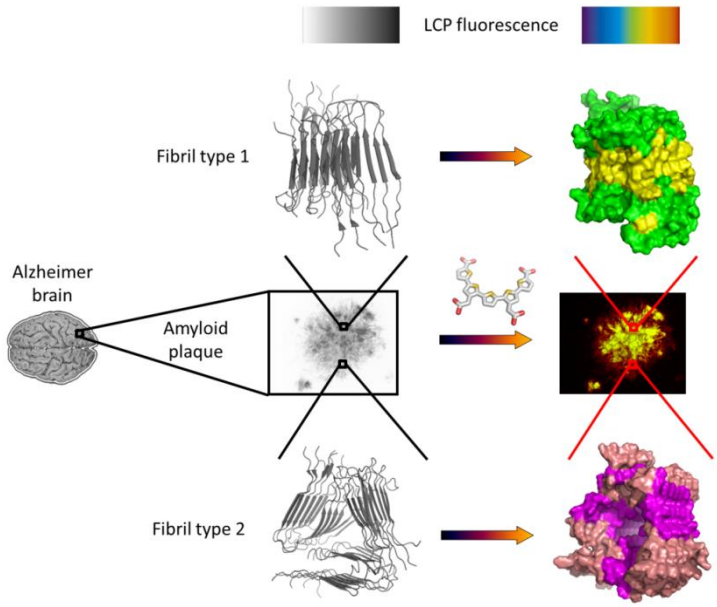


Figure 1: *The principle of LCP fluorescence markers for structural analysis of amyloid fibrils. Different fibril types have different structures and hence different fluorescence from the bound LCP.*

From a library of LCPs, we identified lead compounds with distinct chemical groups for detection and spectral assignment of protein deposits in Alzheimer's and prion diseases. Amyloid structures are vastly heterogenous. It is not known which structures are most relevant for disease. A number of LCPs displayed spectral discrimination of amyloid structures both from the same protein, (PrP, Aβ) as well as for discrimination of different amyloids within the same patient (Aβ, Tau). In addition, we have shown that pre-fibrillar aggregates of PrP, and Aβ (oligomers) that goes undetectable by conventional amyloid ligands are readily detectable by LCPs. The ability of LCPs to discriminate between different structures of fibrillar and pre-fibrillar aggregates is of great importance for Alzheimer's disease. These features render the LCPs superior to other small molecule probes for molecular diagnostics. Importantly, these LCPs can also be used for in vivo imaging of protein deposits in the brain of living animals. Hence, LCPs that are selective amyloid ligands and even capable of crossing the blood brain barrier were developed within the LUPAS project. Synthesis of LCPs were scaled up into gram scale amounts and are now being implemented within a variety of subprojects within LUPAS partner labs and within collaborations all over Europe and worldwide.

Multiphoton imaging is a preferable technique for studying protein aggregation diseases in real time in transgenic mouse models and we have shown that the unique optical properties

of LCPs make these dyes highly efficient for multiphoton *in vivo* imaging. Several LCPs cross the blood brain barrier which enables specific labeling of amyloid plaques (Aβ) and intraneuronal Neurofibrillary tangles (Tau) in the parenchyma of the brain, as well as Aβ amyloid in blood vessels so called cerebral amyloid angiopathy. *In vivo* imaging of protein deposits in living mice was demonstrated using distinct LCPs and these LCP scaffolds are well tolerated by mice even during longer time periods and repetitive injections. The latter is imperative, because the final goal of LUPAS is to achieve an LCP based agent for non-invasive clinical diagnostics of AD and prion diseases.

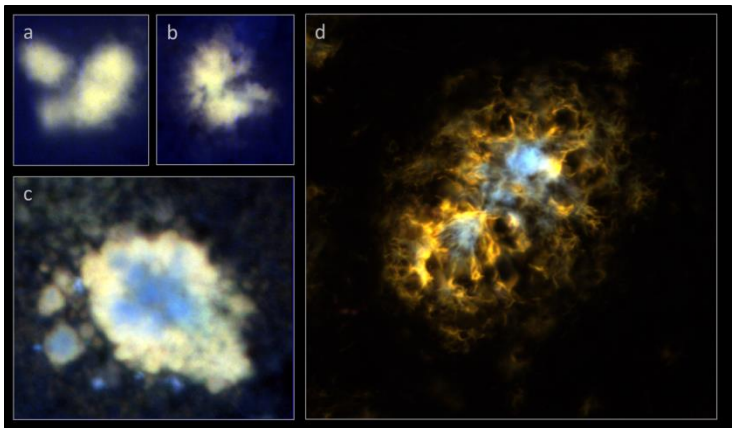


Figure 2: *LCP fluorescence from protein aggregates in frozen brain tissue from human patients with prion disease: a) Creutzfeldt-Jakob Disease (CJD)type I, b) CJD type II, c) Inherited prion disease Gerstmann-Straussler-Scheinker Disease (GSS). d) Amyloid plaque in transgenic mouse with Alzheimer disease pathology.*

From a diagnostic perspective, we have employed the LCPs on post-mortem tissue sections from patients with neurodegenerative diseases. The LCP selectively stain the protein deposits and expose these major pathological hallmarks of either AD or prion diseased brain. The identification can easily be done and distinguished due to the color emitted from the LCP bound to the different entities (**Figure 2**). Hence, the LCP technique shows excellent promise for being implemented as a complementary technique in routine clinical diagnostics. Work towards fluorescence detection of aggregated protein as biomarkers in cerebral spinal fluid of Alzheimer's disease and control patients has been performed, but results so far have not been conclusive.

Optical probes are not the preferable agents for non-invasive imaging in humans, due to the limitations of optical imaging. In this regard, the LUPAS consortium aimed to broaden the scope to develop novel multimodal LCPs that can be used for both optical imaging and magnetic resonance imaging (MRI). MRI is today a standard technique for imaging of pathological conditions in humans, but subtle pathological changes in tissue can be hard to detect due to limited effect on the MR signal. Contrast agents can be used to enhance the

MR image contrast related to tissue pathology. Today only unspecific contrast agents are in use, but targeted contrast agents based on paramagnetic and superparamagnetic nanoparticles and nano-complexes (MNPs) hold great promise for MRI. Within LUPAS we have synthesized a variety of nanoparticles for enhanced T1 and T2 relaxation dispersion potentially useful as contrast agents for MRI. In addition, the first prototype of a LCP-MNP conjugate can specifically target amyloid *in vitro*, in tissue samples, and promising results have also been obtained *in vivo* in transgenic mice.

Therapy

Disease modifying treatments for neurodegenerative diseases are scarce and non-existent for prion diseases. Because the LCP molecules show selective binding towards the culprit – the aggregated proteins their potential as therapeutic agents against prion diseases was evaluated within LUPAS. Work on tissue sections and cell cultures show efficient LCP mediated reduction in prion infectivity as a result of unexpected stabilization of prion aggregates. This mechanistic insight opens up completely new avenues for targeting both prion disease and other neurodegenerative diseases based on hyperstabilization rather than clearance of the misfolded protein culprits.

Description of the main S&T results

Synthesis of luminescent conjugated polymers (LiU)

Monomeric and trimeric precursor thiophene compounds with distinct side functionalities, including a variety of amino acid derivatives, were synthesized. In the initial screen we included two molecules with ethylamine sidechains on thiophene rings 2 and 4 (5006, 5007), however, as indicated in earlier studies we focused on, anionic LCPs containing the monomer 3- thiophene acetic acid which have shown the best selectivity and specificity towards AMTs and protein aggregates in tissue under close to physiological conditions. Therefore our efforts in were focused on developing anionic LCPs with carboxylic acid functionalizations. From a selection of building blocks, we have utilized Suzuki coupling to synthesize a library of chemically defined oligothiophenes, ranging from tetrameric to heptameric thiophenes, having amine or carboxylic acid functionalities at distinct positions along the thiophene backbone (**Table 1**). The synthetic routes for some of the LCPs are rather straightforward (**Figure 1-2**) and have been optimized to render up to gram quantities of individual LCPs.

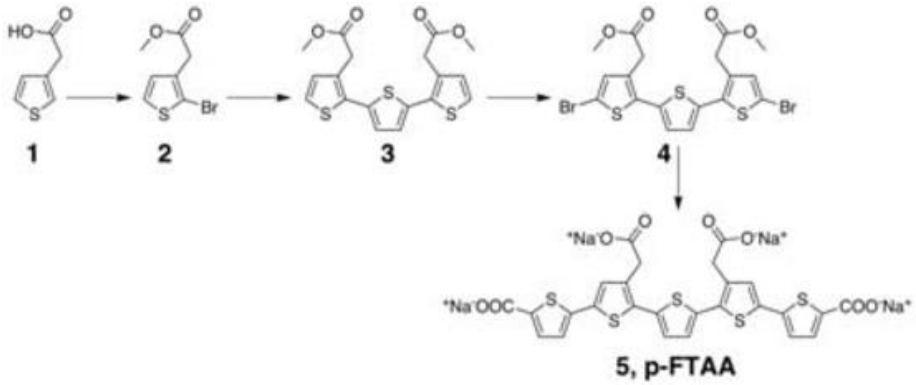
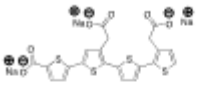
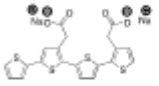
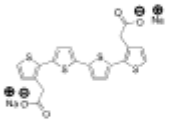
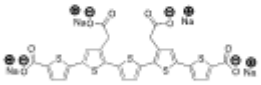
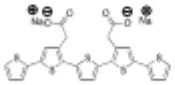
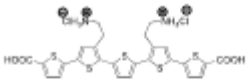
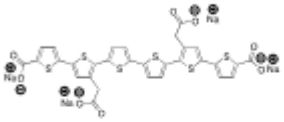
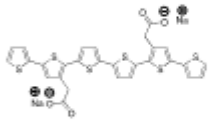
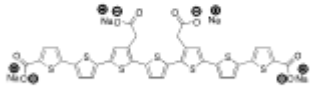
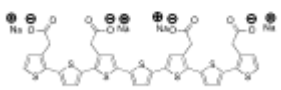


Figure 1: *The straight forward synthesis of p-FTAA (LCP 5001). Details are found in reference X.*

Early on in LUPAS we published a detailed protocol on the synthesis of p-FTAA (**Figure 1**). The straightforward synthesis of p-FTAA, including two Suzuki couplings makes this a highly tractable molecule for many labs with adequate synthetic expertise.

The method was published on the Nature Protocols website: "Synthesis of a pentathiophene fluorescent probe, 4',3'''-bis-carboxymethyl-[2,2';5',2'';5'',2''';5''',2''''']quinquethiophene-5,5'" by Andreas Åslund, K. Peter Nilsson & Peter Konradsson Protocol Exchange doi:10.1038/nprot.2010.24 Nature Protocols website Community contributed.

Table 1: Selection of LCPs synthesized in LUPAS

LCO number	LCO name	MW	Structure
4001	q-FTAA	556,5	
4002	q-HTAA	490,6	
4003	t-HTAA	490,6	
5001	p-FTAA	704,7	
5002	p-HTAA	572,7	
5003	p-Jeff	652,7	
5006	p-HTEA	571,7	
5007	p-FTEA	659,7	
6001	hx-FTAA	786,8	
6002	hx-HTAA	654,8	
7001	h-FTAA	868,9	
7002	h-HTAA	897,0	
10001	PTAA	2500,0	

During LUPAS more than 20 novel anionic LCPs (selection listed in **Table 1**) were synthesized whereof several have been synthesized in gram scales. The LCPs have been used throughout the project and have been implemented at all partner sites. The synthetic routes to the mainly employed LCPs, the well-defined anionic LCOs, are shown in Figure 2.

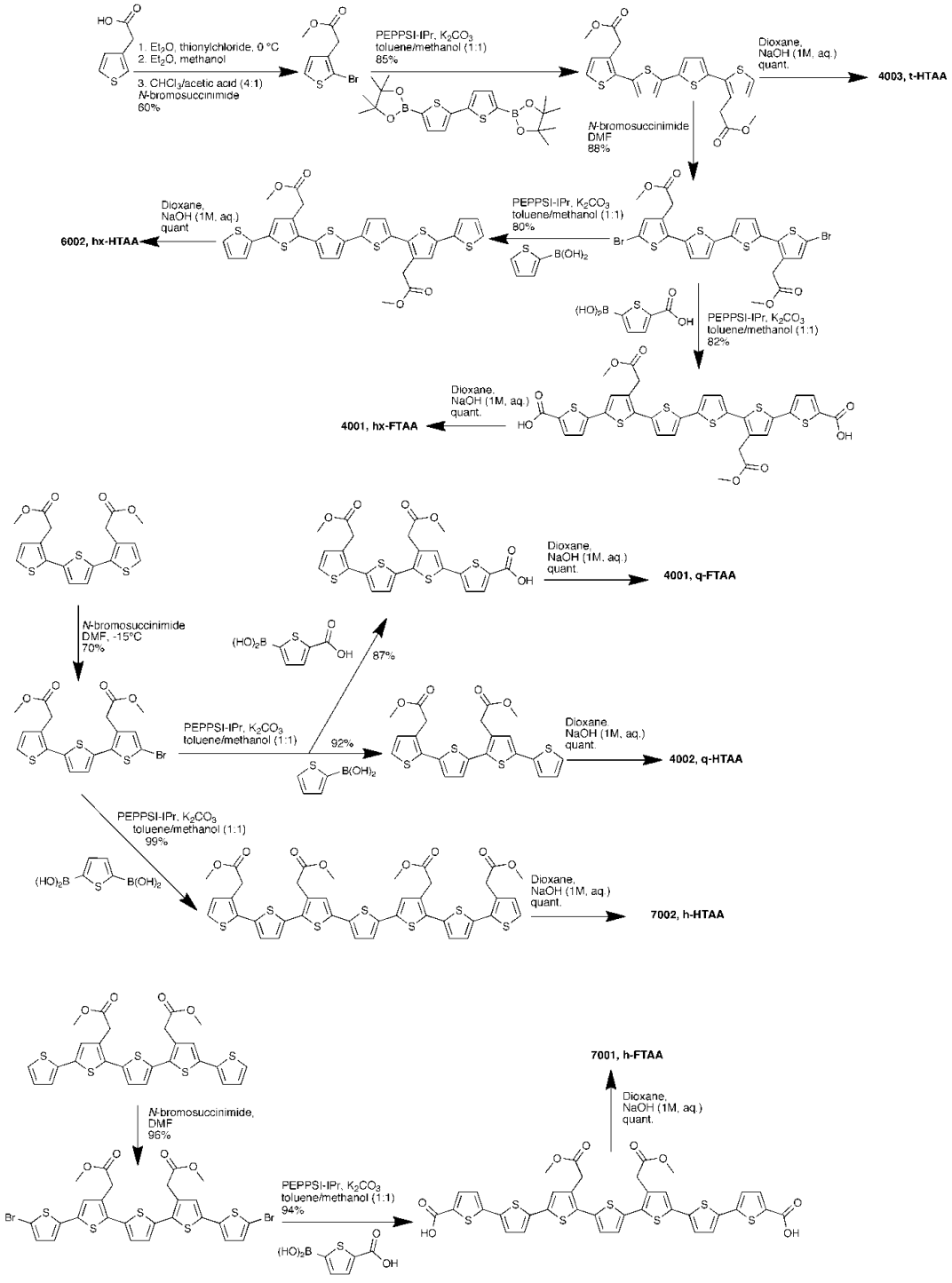


Figure 2: *Schematic representation of the synthetic routes for some of the novel LCPs.*

Multifunctional amyloid specific LCP-nanoagents for MRI and fluorescence

The concept of enhancing the detection methodology of amyloid towards in vivo imaging using magnetic resonance imaging requires paramagnetic species as contrast agents. Smart contrast agents with amyloid target specificity are conceptually shown in **Figure 3**.

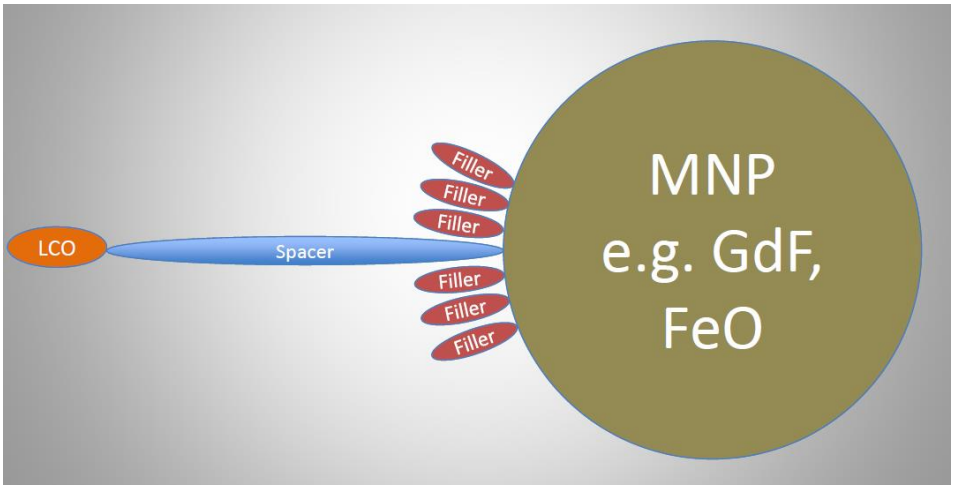
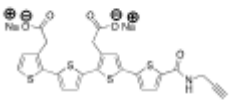
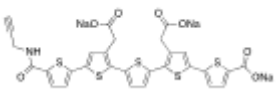


Figure 3: *The conceptual configuration of magnetic nanoparticle (MNP) and LCP amyloid imaging agents. The molecular complex contains 4 parts: LCO for amyloid specificity and fluorescence, spacer, filler for encapsulation and biocompatibility and MNP for magnetic resonance contrast.*

Within LUPAS the amyloid specific anchor was based on LCP molecules. For magnetic nanoparticle (MNP) covalent attachment, asymmetrical variants of the anionic LCPs have also been synthesized. These amyloid specific ligands will anchor the MNP to amyloid plaque. Two lead LCPs are shown in Table 2.

Table 2: Selection of asymmetrical LCP scaffolds for MNP attachment

LCO number	LCO name	MW	Structure
4004	q-prop-FTAA	571,6	
5008	p-prop-FTAA	719,7	

A variety of spacer chemistry both in terms of chain length and composition was developed in LUPAS. One promising spacer and the synthetic scheme for generation of spacer and LCP linkage is shown in **Figure 4**.

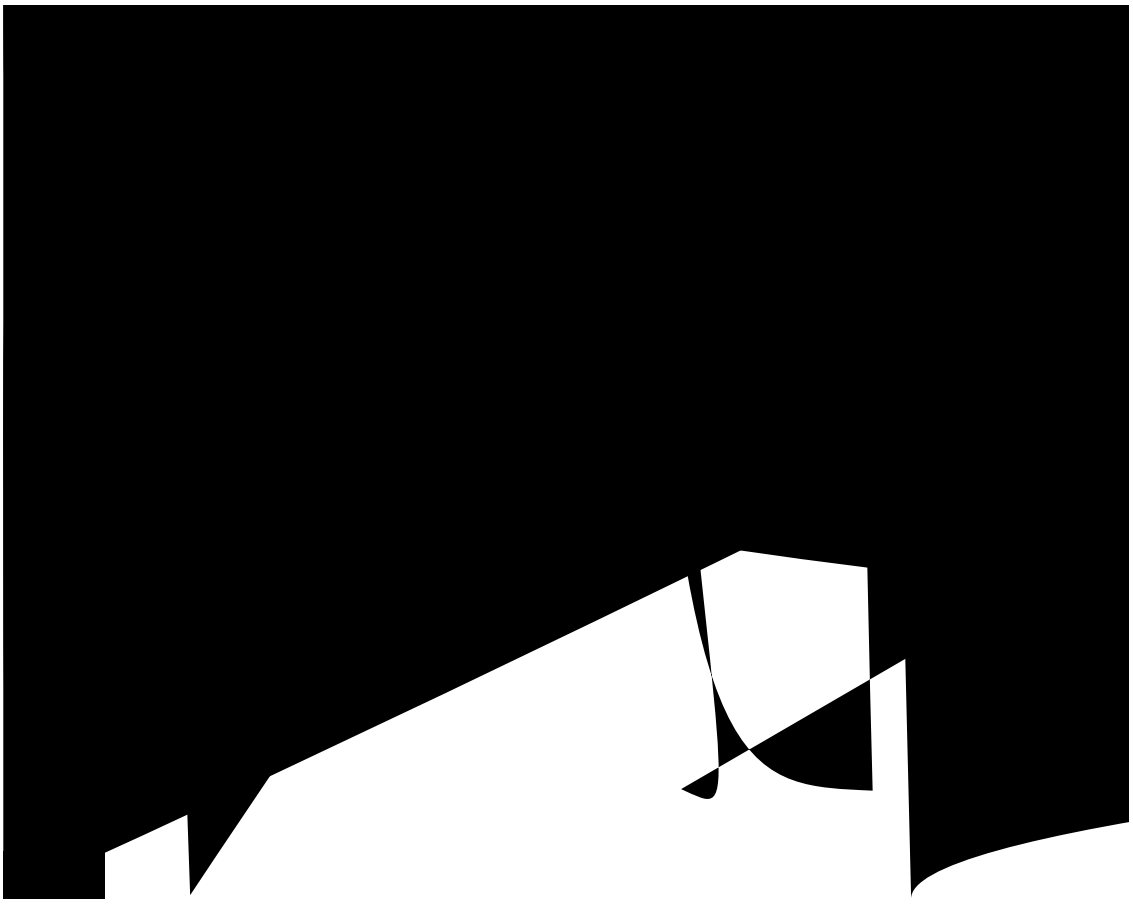


Figure 4: *Spacer synthesis and linkage to LCP for further attachment to MNPs.*

Nano synthesis

Nanoparticles are interesting platforms for such application since it concentrates magnetic ions in a limited volume and is susceptible to bear functional groups. In this work package the investigation on several types of nanomaterials based on Fe, Gd or Mn are performed (Figure 3.). The influence of the chemical composition, size, and shape of the particles on the relaxivities and NMR properties was checked.

Nano particles for relaxivity enhancement in MRI

Magnetic resonance imaging (MRI) is a non-invasive method that allows for longitudinal imaging of animals and patients. In dedicated animal MR-scanners, the desired organ or body part, for example the brain in rats and mice can be imaged in vivo with high quality and resolution. Spatial resolution in MRI is dependent on the signal to noise ratio (SNR) and the two main factors are image acquisition duration and magnetic field strength. When the object is put into the MR-scanner, all the protons within the object act as small magnets and

align along the magnetic field inside the MR-scanner spinning around the axis of the magnetic field. A short excitation pulse is then applied, shifting the alignment of the proton spins out of this direction. After the excitation pulse comes to an end, a signal is recorded in a coil. The signal created by the proton spins decays thereafter by two relaxation processes (called T1 and T2 relaxation) as the protons *relax* back to their original alignment. T1 and T2 vary with the tissue type, and by weighting the images by the T1 and T2 contrast can be created in the MR images. Commonly used contrast agents such as Gadolinium- or ironoxide complexes can be used to alter the relaxation properties of the tissue, leading to increased or reduced signal from the tissue where the contrast agents are present. Within LUPAS the relaxivity of 20 different particles have been measured on different field strength and 6 different conjugated MNP-LCPs have been measured at 7T. The relaxivity measurements indicate that the main properties of the particles are kept or enhanced after conjugation to LCP with different linkers. This is due to that most of the promising particles have high r2 relaxivity, and increased size will increase r2.

LCP-MNP retains amyloid fibril specificity

That the selective property of amyloid targeting was retained in LCP-MNP complexes was assessed by both fluorescence and electron microscopy of in vitro generated AMTs from Aβ1-42 (**Figure 5**).

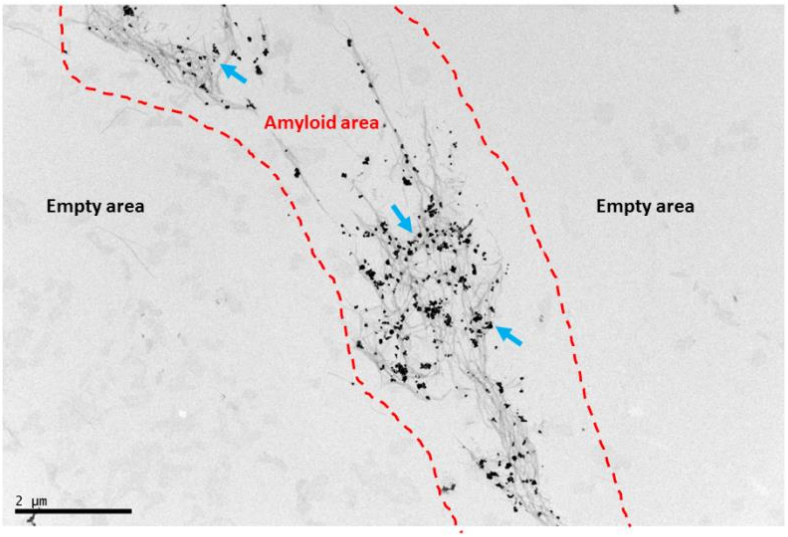


Figure 5: *LCP-MNP binds to Aβ1-42 amyloid fibrils in vitro. The main portion of the TEM grid is empty. Within the amyloid fibril covered area MNP-LCP particles are visible as dark clusters bound to the fibrils.*

Development of Amyloidotic Molecular Targets (AMTs) of Aβ and PrP for LCP screening

For screening of suitable photophysical LCP properties and binding parameters of LCPs we employed amyloidotic molecular targets (AMTs) generated in vitro by recombinant protein preparations. Analyses of preparations were made both after reaching equilibrium and during kinetic measurements. The AMTs used were based on Aβ preparations and PrP employing quiescent and shaking conditions as well as different Aβ1-40 and Aβ1-42 mixtures in defined ratios. The spectroscopic screening with LCPs with AMT material was used for optimal settings for microscopy as well as for selection of reference spectra for tissue sections. From the kinetic screen we discovered that p-FTAA was a highly suitable molecule for generating LCPs sensitive for non-mature (ThT negative) prefibrillar amyloid, both from Aβ and human prion protein as well as other proteins (**Figure 6**). This finding was published in Biochemistry 2010. "A fluorescent pentameric thiophene derivative detects in vitro-formed prefibrillar protein aggregates" Hammarström P, Simon R, Nyström S, Konradsson P, Aslund A, Nilsson KP. Biochemistry. 2010 Aug 17;49(32):6838-45.

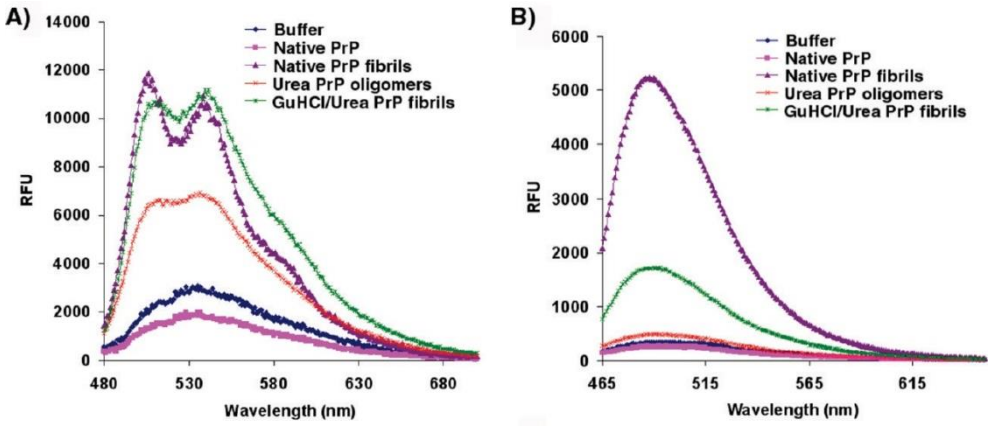


Figure 6: *p-FTAA (A) and ThT (B) fluorescence from different in vitro preparations of recombinant human PrP 90-231. Notably p-FTAA identified oligomeric PrP aggregates (red curves) that were not observed by ThT fluorescence. Figure adapted from Hammarström et al., 2010.*

A focused library of LCPs followed this initial lead and allowed us to conclude that this feature of detection of prefibrillar aggregates is shared by anionic LCPs which should have a backbone consisting of five to seven thiophene units and carboxyl groups extending the conjugated thiophene backbone (Klingstedt et al. 2011) (**Figure 7**). Importantly the carboxyl moiety extending the conjugation of the thiophene backbone also allowed for spectral discrimination of Aβ and Tau within human tissue samples.

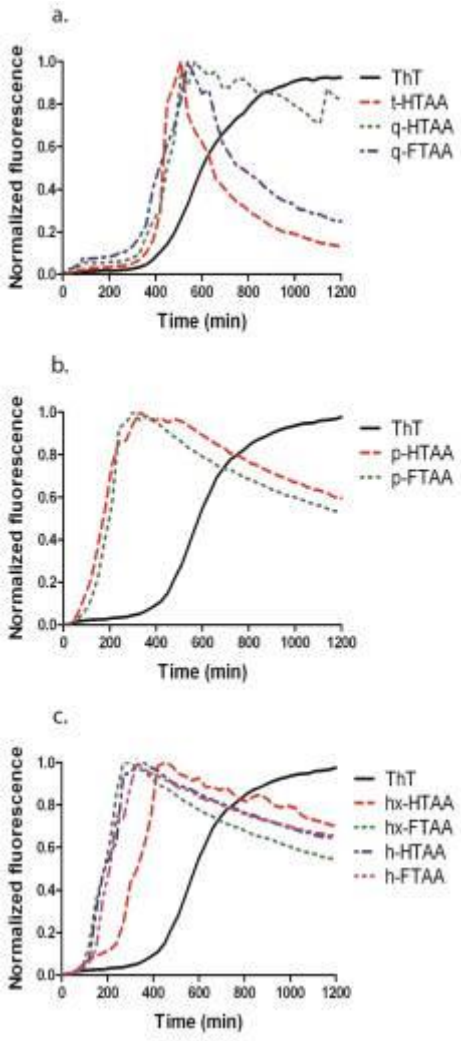


Figure 7*: Fibrillation of recombinant Aβ1-42 monitored by fluorescence from ThT or LCOs. (a) Time plot showing the fibrillation kinetics of Aβ1-42 monitored by fluorescence from ThT (black solid line), t-HTAA (red dashed line), q-HTAA (green dotted line) or q-FTAA (blue dot-dashed line). (b) Time plot of Aβ1-42 fibrillation kinetics monitored by fluorescence from ThT (black solid line), p-HTAA (red dashed line) or p-FTAA (green dotted line). The pentamers detect early non-thioflavinophilic species in the fibrillation pathway and showed initiation of a growth phase already after 60 minutes. (c) Time plot of Aβ1-42 fibrillation kinetics monitored by fluorescence from ThT (black solid line), hx-HTAA (red dashed line), hx-FTAA (green dotted line), h-HTAA (blue dot-dashed line) or h-FTAA (magenta dot-dot-dashed line). Both hexamers and heptamers detected non-thioflavinophilic species in the fibrillation pathway and showed initiation of the growth phase around 90 minutes. Figure adapted from Klingstedt et al., 2011.*

The library design and conclusions from the AMT screen was published in Organic and Biomolecular Chemistry 2011. “Synthesis of a library of oligothiophenes and their utilization as fluorescent ligands for spectral assignment of protein aggregates”. Klingstedt T, Aslund A,

Simon RA, Johansson LB, Mason JJ, Nyström S, Hammarström P, Nilsson KP. Org Biomol Chem. 2011 Dec 21;9(24):8356-70.

Development of AMTs for human prion protein instead of chemical preparations (**Figure 6**) also entailed protein engineering. Within LUPAS we investigated the molecular basis for the dependency for misfolding of PrP as a consequence of variations in position 129. The 129 site is well known to be highly important for disease penetrance and clinical presentation, being either M (wt) or V (variant). Our data based on the *in vitro* behavior of PrP in solution, showed that position 129 was an interesting site for recruitment of PrP into the amyloid state. Charge mutations (M129E and M129K) showed decreased spontaneous conversion propensity (nucleation) compared to the wild type. We also observed that M129C was totally resistant towards spontaneous engulfment of PrP into the amyloid fold, most likely due to the formation of an intermolecular disulfide bridge forming in the lag phase, rendering a non-convertible disulfide linked dimer effectuated by the 129CC interaction. In contrast to the rather large differences in fibrillation propensity found for extreme amino acid substitutions such as tryptophan, proline, cysteine and charge, quite minor changes were observed for conservative mutations M129L, M129A and as expected M129V. From these data we have formulated a molecular platform for experimental AMT assessment (**Figure 8**) of e.g. synthetic prions and targets for LCP screening.

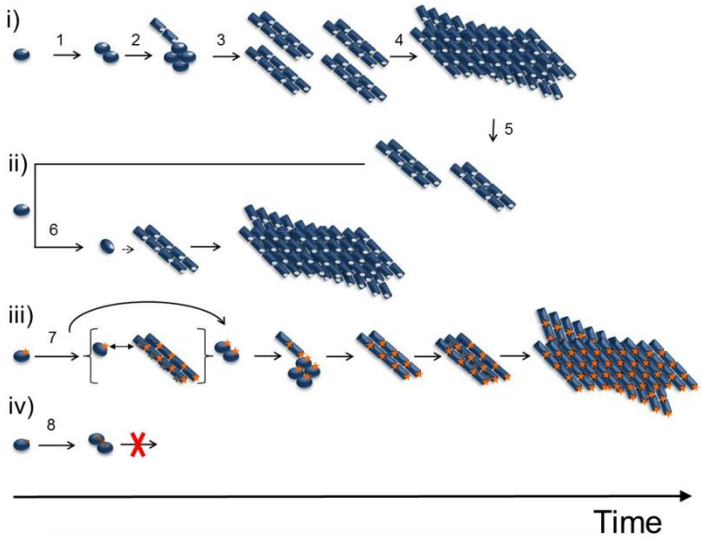


Figure 8: *Schematic mechanisms and kinetics of PrP fibril formation evaluated by amino acid substitutions in position 129. i) Spontaneous fibrillation in the general case. Native HuPrP (position 129 marked in gray) forms a native dimer (1) which is further converted into a fibrillation competent misfolded oligomeric aggregate (2). This conformational rearrangement is stabilized by intermolecular interactions and exposes different surface amino acids than the native protein. The protein further converts into fibrillation nuclei (3) and fibril elongation is initiated (4), followed by*

fibril fragmentation (5) in the exponential growth phase. ii) Seeded reaction. The fibrillation nuclei can directly recruit and convert monomers into the fibrillar state (6).iii) In the case of 129K, marked in orange, the recruitment of native monomer to the nuclei is abrogated by positive-charge repulsion caused by charged residues exposed on the surface of the fibrillation competent conformation. The seeding mechanism is distorted. The spontaneous reaction is also to some extent delayed by positive charge repulsion (7). iv) For 129C the formation of a native dimer enables intermolecular covalent disulfide formation (8) and conversion into fibrillation competent conformation is blocked.

This study was published in The Journal of Biological Chemistry 2012. "Multiple substitutions of methionine 129 in human prion protein reveal its importance in the amyloid fibrillation pathway" Nyström S, Mishra R, Hornemann S, Aguzzi A, Nilsson KP, Hammarström P. J Biol Chem. 2012 Jul 27;287(31):25975-84.

Use of LCPs for animal and human samples

Luminescent conjugated polythiophenes (LCPs) with molecular scaffolds consisting of repetitive thiophene moieties have evolved as an interesting class of fluorescent probes, since this class of molecules offers the possibility to achieve an optical read-out of biomolecular interactions through their impact on the conformation and the geometry of the LCP backbone. These structurally induced optical changes of the LCP backbone also allows the tantalizing possibility to detect conformational changes of aggregates, rendering opportunities to gain more information concerning the morphology of the protein deposits. The use of LCPs have been reviewed both for versatile and detailed usage by LUPAS researchers in Methods in Molecular Biology, 2012, "A pentameric luminescent-conjugated oligothiophene for optical imaging of in vitro-formed amyloid fibrils and protein aggregates in tissue sections". Nilsson KP, Lindgren M, Hammarström P. Methods Mol Biol. 2012;849:425-34 and Biochemical Society transactions 2012, "Luminescent conjugated poly- and oligo-thiophenes: optical ligands for spectral assignment of a plethora of protein aggregates". Klingstedt T, Nilsson KP. Biochem Soc Trans. 2012 Aug;40(4):704-10.

LCPs for Alzheimer's disease

It is currently recognized that amyloid deposits *in vivo* are vastly heterogenous and appear polymorphic. LCPs have provided the opportunity to gain more information concerning the morphology of the protein deposits, and a greater understanding of the conformational phenotype encoded in distinct protein aggregates should be achieved. Instead of merely observing the total amount of protein aggregates, the LCPs allow spectral finger printing of heterogenic population of specific protein aggregates and these ligands have been efficient for studying protein aggregates in transgenic mouse models having AD Aβ pathology. This was one of the core aims for LUPAS.

Tissue sections mainly from mouse brain were herein stained with a variety of LCP probes and were used to assay amyloid binding, specificity and spectral features, mainly using different microscopy based techniques (**Figure 9**).

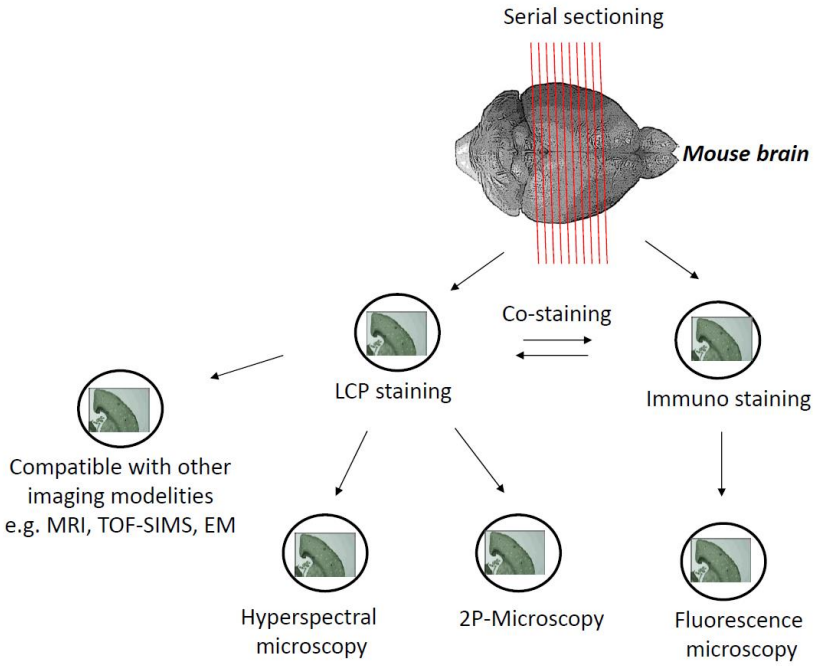


Figure 9: *Overview of LCP applications using microscopic studies of mouse brain tissue. Serial sectioning affords samples which can be solely stained with LCP or in combination with other fluorophores including immunofluorescence. LCP stained sections can be alnalyzed by 2P-microscopy or hyperspectral microscopy, and is compatible with other imaging modelities such as Magnetic resonace imaging, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and electron microscopy.*

Several amyloid pathological hallmarks of disease were studied in AD mouse models APP/PS1 and APP23 mice aged 18 months. Most studied LCPs identified amyloid plaque and cerebral amyloid angiopathy (CAA) (**Figure 10**) The robustness of staining was also verified after prolonged storage of tissue sections (in the dark) by revisiting the sections after 2 years (**Figure 10**).

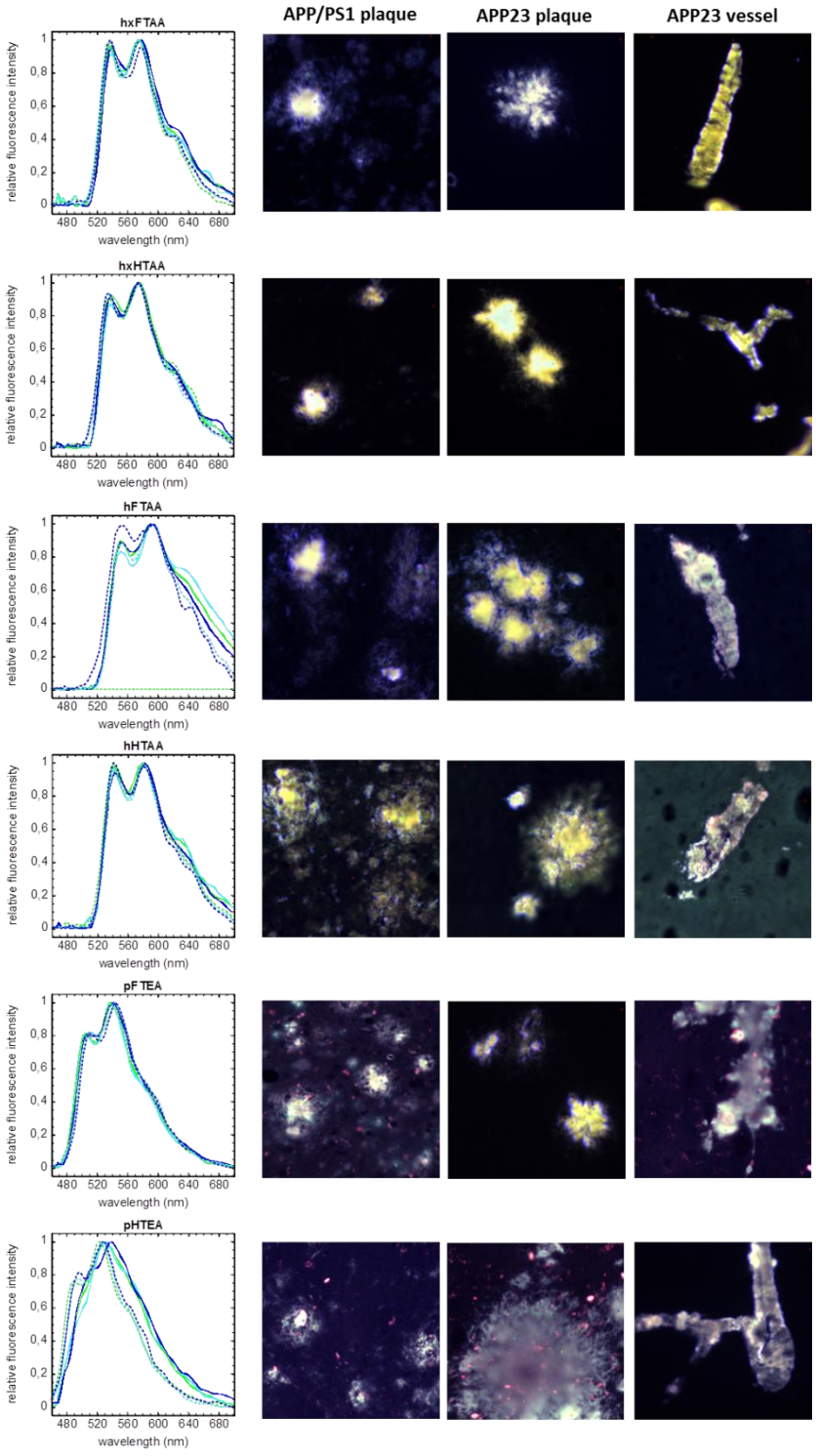


Figure 10: *Fluorescence micrographs and spectra from the different amyloid types and LCPs. APP/PS1 plaque ----- APP23 plaque ----- APP23 CAA ----- Continuous line = spectrum from freshly stained tissue, broken line = spectrum from the same tissue two years after staining.*

Compatibility of LCPs with other imaging modalities

Importantly the LCP molecules are highly compatible with new imaging technologies. In one study the LCP imaging tracer pFTAA was used for combined imaging using fluorescence tracing of amyloid plaque and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) was used to investigate cholesterol accumulations originating from surrounding astrocytes proximal to plaque (**Figure 11**). This study was recently published in Acta Neuropathologica 2013, ”Localization of cholesterol, amyloid and glia in Alzheimer's disease transgenic mouse brain tissue using time-of-flight secondary ion mass spectrometry (ToF-SIMS) and immunofluorescence imaging” Solé-Domènech S, Sjövall P, Vukojević V, Fernando R, Codita A, Salve S, Bogdanović N, Mohammed AH, Hammarström P, Nilsson KP, LaFerla FM, Jacob S, Berggren PO, Giménez-Llort L, Schalling M, Terenius L, Johansson B. Acta Neuropathol. 2013 Jan;125(1):145-57.

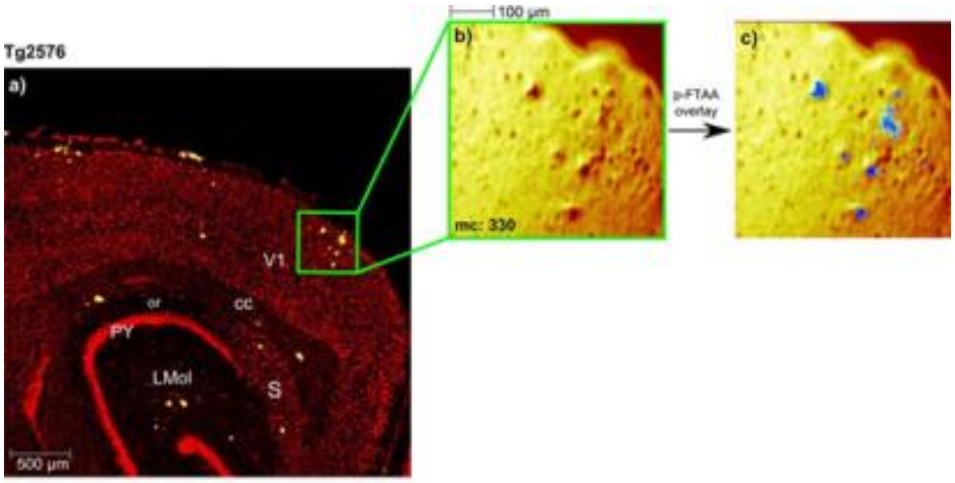


Figure 11: *Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and confocal laser scanning fluorescence microscopy.images of areas occupied by Aß deposits in mouse brain tissues from a Tg2576 mouse.* ***(a)*** *Brain section stained with p-FTAA (amyloid, yellow) and BOBO-1 (cell nuclei, red).* ***(b)*** *ToF-SIMS image of the area delineated by a green square in (a), showing dark regions with very low signal intensity, appearing as topographic protrusions on the tissue surface.* ***(c)*** *Overlaid p-FTAA TOF-SIMS images demonstrating that the multiple surface protrusions in the (b) co-localize with Aß deposits.*

In vivo imaging

Repetitive in vivo imaging in mice is an important tool for studying dynamic changes in structure and function of the brain. A methodology by LUPAS researchers describes a head fixation system, which allows rapid re-localization of previously imaged regions of interest (ROIs) within the brain. Such ROIs can be automatically relocated and imaged over weeks to months with negligible rotational change and only minor translational errors. Previously

stored imaging positions can be fully automated re-localized within a few seconds (**Figure 12**). This automated rapid and accurate relocation simplifies image acquisition and post-processing in longitudinal imaging experiments. The risk of laser induced phototoxicity is greatly reduced, because the laser is only used for data acquisition and not for finding previously imaged ROIs. Thus, here described head fixation device appears well suited for in vivo repetitive long-term imaging in mouse brain.

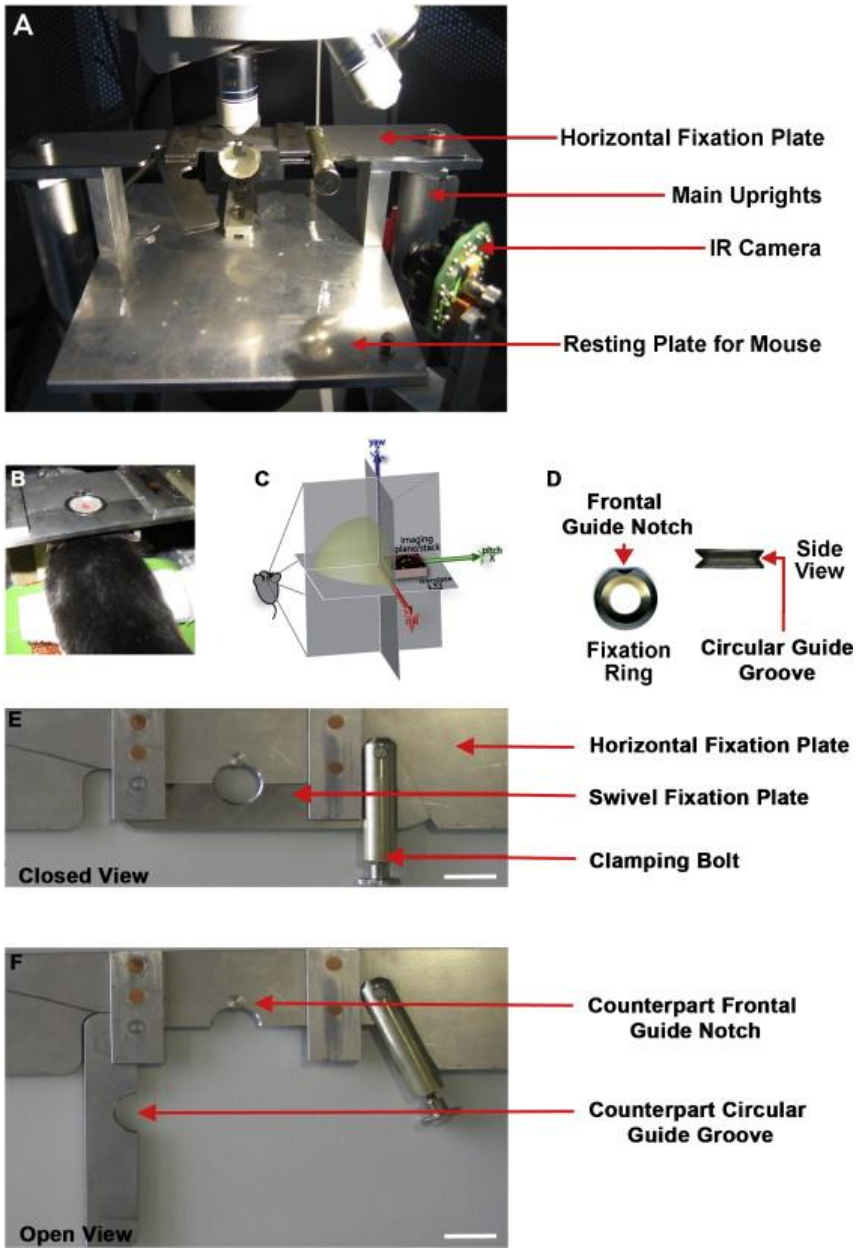


Figure 12: (A) *Fully constructed fixation device under the 2-photon microscope with an infrared camera used to monitor the animal during the imaging progress. (B) Mouse with titanium ring fixed to the horizontal fixation plate under the 2-photon microscope. (C) The guide notch and groove of the titanium ring and the respective counterparts of the horizontal fixation plate reduce tilting in all 3*

dimensions (yaw, pitch and roll). (D) Titanium ring for fixation to mount onto the mouse (top and side views). (E) Main horizontal plate for fixation (closed (E) and open (F) view; scale bar = 15 mm). The construction can be done with standard metal shop equipment (lathe/milling/saw), and reasonable time/material expenditures. Figure adapted from Hefendehl et al., 2012.

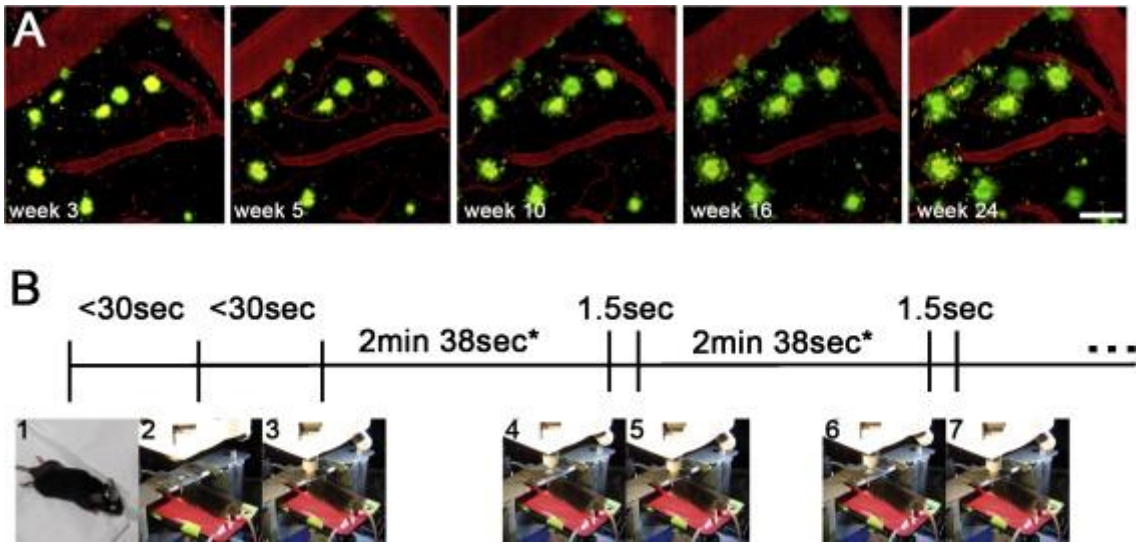


Figure 13: *(A) Long-term imaging of APPPS1 transgenic mice for up to 25 weeks (Hefendehl et al., 2011). Same region of interest is shown with amyloid deposits (green) stained with methoxy-X04, and blood vessels (red) made visible by Texas Red® dextran. A maximum z-projection is shown for the imaged volume including truncated plaques located at the rim of the volume. Scale bar 50 µm. (B) Timeline of imaging session including head fixation and re-localization of ROIs. (1) An anesthetized animal can be fixed under the 2-photon microscope in under 30 s (n = 10). (2) Adjusting the right objective and re-localization of a previously stored position can be done in under 30 s. (3) Imaging a z-stack of 100 µm (*100 Hz, bi-directional, Format 1024 × 1024, z-step 1 µm) is performed in 2 min and 38 s. (4) Automatic re-location to the next previously stored ROI takes 1.5 s on average (n = 12). Figure adapted from Hefendehl et al., 2012*.

The initial work of the above mentioned technology has been used to study Aβ-amyloid plaque formation and growth over time (Figure 13). Plaque growth in APP/PS1 were shown to grow radially by 0.3 µm per week (Hefendehl et al., 2011). The tracer used in these experiments were the methoxy-X04 dye developed by the Klunk laboratory (Klunk WE et al. J Neuropathol Exp Neurol. 2002 61(9):797-805), but can also be performed using LCPs. These developments and discoveries were published in two papers in Journal of neuroscience and Journal of Neuroscience Methods. “Long-term in vivo imaging of β-amyloid plaque appearance and growth in a mouse model of cerebral β-amyloidosis”, Hefendehl JK, Wegenast-Braun BM, Liebig C, Eicke D, Milford D, Calhoun ME, Kohsaka S, Eichner M, Jucker M. J Neurosci. 2011 Jan 12;31(2):624-9; “Repeatable target localization for long-term in vivo imaging of mice with 2-photon microscopy”, Hefendehl JK, Milford D, Eicke D, Wegenast-

Braun BM, Calhoun ME, Grathwohl SA, Jucker M, Liebig C. J Neurosci Methods. 2012 Apr 15;205(2):357-63.

In vivo imaging using LCPs

Initial experiments from LUPAS researchers revealed that LCPs pFTAA (5001) and pHTAA (5002) showed efficient penetrance of the BBB and readily stained amyloid plaque in APP/PS1 mice (Åslund 2009). Following this success within LUPAS we employed the larger molecule hFTAA (7001), which has a more red shifted fluorescence spectrum and equal or superior sentitivity than pFTAA. The LCP hFTAA passed the blood-brain barrier after systemic administration and was specifically bound to extracellular β-amyloid deposits in the brain parenchyma (Aβ plaques) and in the vasculature (cerebral β-amyloid angiopathy) of β-amyloid precursor protein transgenic APP23 mice (**Figure 14**). Interestingly, peripheral application of hFTAA also stained intracellular lesions of hyperphosphorylated Tau protein in P301S Tau transgenic mice (**Figure 14**). In addition hyperspectral imaging of all three amyloid types was acquired ex vivo using two-photon excitation. Within this work an algorithm was developed to demonstrate the LCP ability to distinguish between two amyloid lesions and the background. hFTAA revealed a distinct shift in its emission spectra when bound to Aβ plaques versus Tau lesions. A spectral difference was also observed for Aβ plaques versus cerebral β-amyloid angiopathy, indicating that different amyloid types and structural variances of a specific amyloid type can be distinguished. Hence, by adding spectral signatures to amyloid lesions, our results pave the way for a new biophotonic era of *in vivo* amyloid imaging, allowing in vivo differentiation of amyloid polymorphisms and monitoring changes of their structure/composition over time.

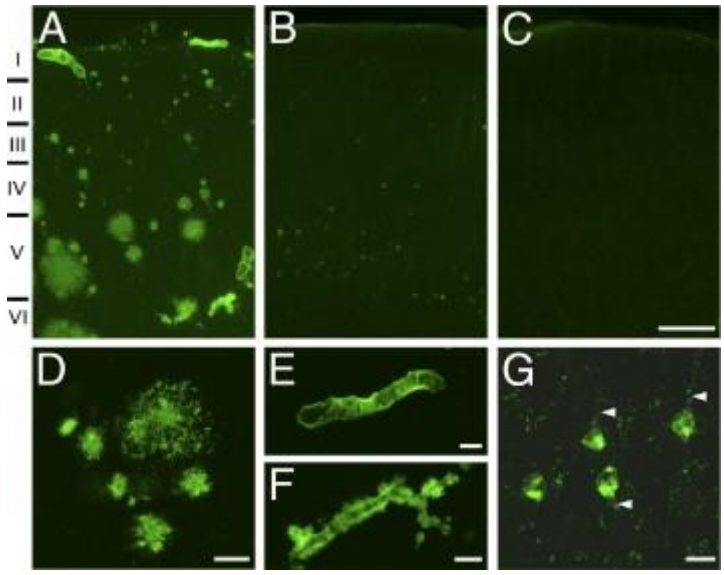


Figure 14: *The LCP hFTAA (7001) passes the BBB and binds to amyloid lesions. At 24 hours after a single i.v. injection of hFTAA, characteristic amyloid lesions are labeled throughout the brain of*

transgenic mice. ***A:*** *APP23 transgenic mice depict staining of Aβ plaques and CβAA. Imagery shows hFTAA staining in the cortex of a 21-month-old mouse.* ***B:*** *P301S Tau transgenic mice reveal staining of intracellular Tau aggregates. Cortical staining shows hFTAA-positive cells mainly in layers II and V. As one example, a 6-month-old homozygous mouse is shown.* ***C:*** *Wild-type animals depict no staining.* ***D–G:*** *High-magnification images of the different hFTAA-stained amyloid lesions. hFTAA staining of Aβ plaques (**D**), CβAA of different severity grades (**E** and **F**), and intracellular Tau aggregates (**G**). Protruding neurites are labeled (**arrowheads** in **G**). Small punctate staining in **G** reflects an unspecific autofluorescence signal of lipofuscin. Images in **E–G** are maximum-intensity projections. Scale bars: 200 µm (**A–C**); 50 µm (**D** and **F**); 20 µm (**E** and **G**). Figure adapted from Wegenast-Braun et al., 2012.*

This study was recently published in American Journal of Pathology 2012, "Spectral discrimination of cerebral amyloid lesions after peripheral application of luminescent conjugated oligothiophenes", Wegenast-Braun BM, Skodras A, Bayraktar G, Mahler J, Fritschi SK, Klingstedt T, Mason JJ, Hammarström P, Nilsson KP, Liebig C, Jucker M. Am J Pathol. 2012 Dec;181(6):1953-60.

LCP-MNP retain amyloid plaque specificity ex vivo

The developed LCP-MNP for MRI imaging was initially found to retain amyloid specificity against AMTs in vitro (**Figure 5**). A more complex sample such as tissue samples could post more challenging. Nevertheless, LCP-MNPs were highly fluorescent and showed specific binding and strong fluorescence when bound to amyloid plaque in tissue sections from transgenic mice (**Figure 15**). The specificity and selectivity in these samples were independent of attachment to MNPs reflecting the same fluorescence profiles as the corresponding LCP-spacer adducts (**Figure 15**).

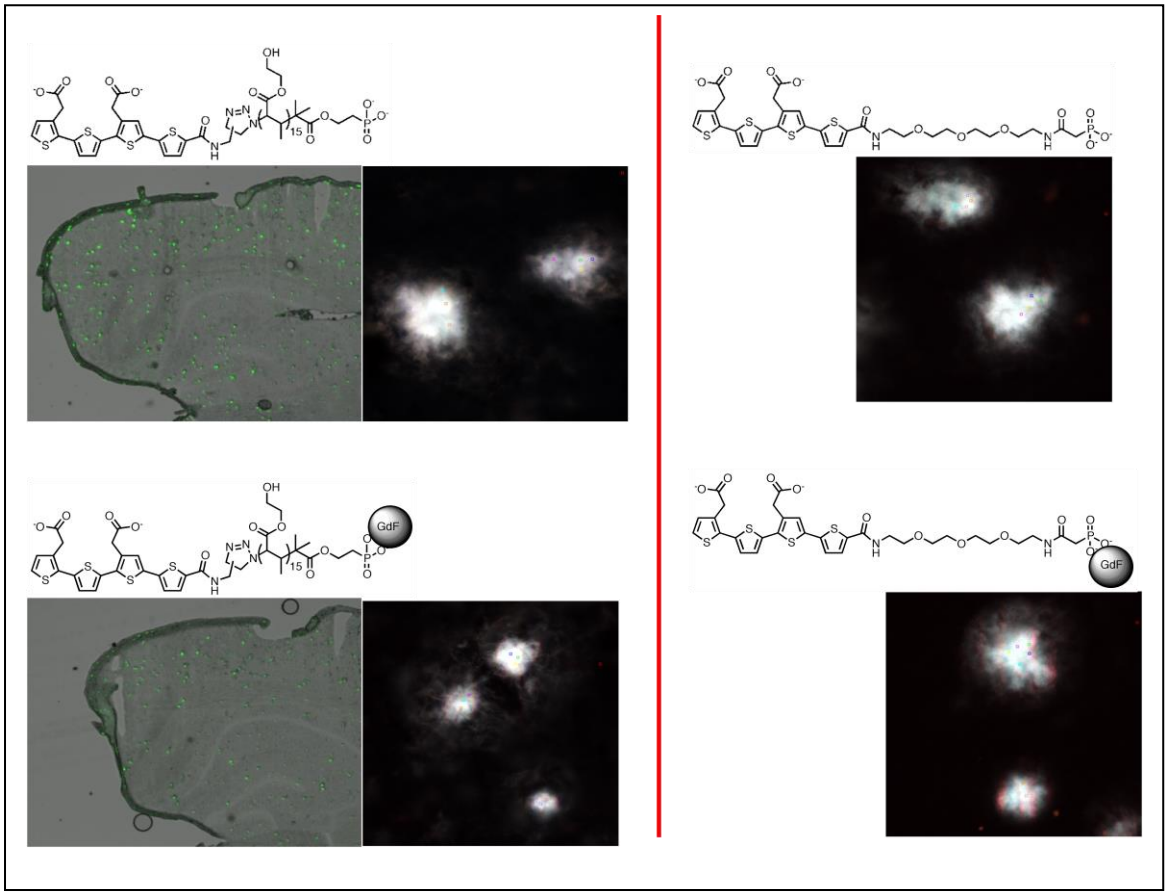


Figure 15: *Amyloid imaging in brain sections of transgenic APP/PS1 mice using LCPs with linkers and conjugated to MNPs. The overview images (left) show green fluorescent plaque which have been targeted by the LCP-linker or LCP-linker-MNPs. Higher magnification micrographs show the enhanced fluorescence and virtually invisible background in plaque rich areas.*

MRI data acquisition *in vivo* in APP/PS1 mice proved more challenging than *in vivo* fluorescence. Several application procedures and LCP-MNP contrast agents showed small amounts of penetrance of LCP-MNPs over the BBB following intravenous (i.v.) inoculations. Better success was achieved with direct administration of the contrast agents into the CSF.

Development of hyperspectral correlation analysis of multicolor, autofluorescence and LCP stained amyloid plaques

Multiphoton imaging is a preferable technique for studying protein aggregation diseases in real time in transgenic mouse models and within LUPAS it was further evident that the unique optical properties of LCPs make these dyes highly efficient for multiphoton *in vivo* imaging. In certain aspects there nevertheless appear problematic with autofluorescence. Green autofluorescence from amyloid plaques can be a problem in weakly stained samples, such as samples that have been i.v. injected at low levels e.g. LCP-MNP probes at low levels of LCP loading <30 µM (**Figure 16**). Also, to analyse multistaining experiments, one needs

special attention to assess the spectral content. Researchers within LUPAS therefore developed a special algorithm that can accurately calculate the spectral similarity of a measured spectrum with a reference spectrum. The method is based on correlation, where the measured spectrum (X) of each pixel in a hyperspectral image is correlated with a reference spectrum (Y), using the following formula:

$$\rho_{XY} = \frac{\sum_{i=1}^{N}(X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{N}(X_i - \bar{X})^2}\sqrt{\sum_{i=1}^{N}(Y_i - \bar{Y})^2}},$$

Where X_i is the i:th wavelength point and the bar over X denotes the average of the spectrum (and similar for Y). This makes it possible to give a mathematical number in the range 0 →1 where 1 is a measured spectrum identical to the reference. The formula is robust to background noise and does not suffer from the same problems as other linear spectral unmixing algorithms.

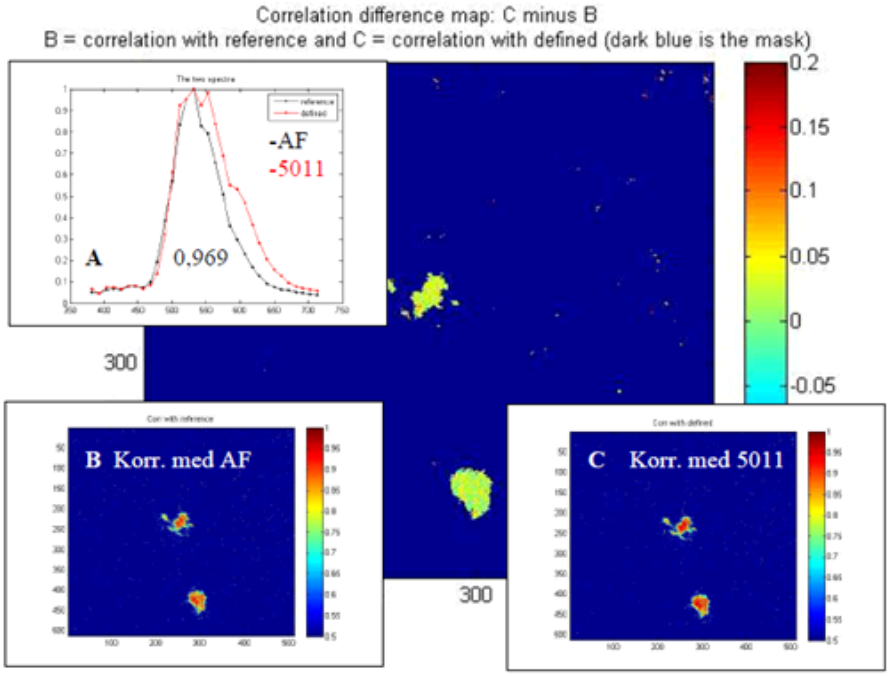


Figure 16. *Spectral correlation analysis of fluorescence spectra of an MNP-LCP i.v. injected (corresponding to 30 µM of LCP) in APP/PS1 mouse, where minor amounts of material reached the amyloid plaque and autofluorescence becomes significant. Herein spectral correlation difference maps could delineate signal from background.*

The method of spectral correlation was recently published in "Hyperspectral analysis using the correlation between image and reference". PG Ellingsen, NK Reitan, BD Pedersen, M Lindgren J. Biomed. Opt. 2013. 18 (2), 020501.

Human Alzheimer's disease (AD) diagnostics

Human brain tissue samples of sporadic AD patients were analysed within LUPAS. Tissues were screened with the anionic LCP library and from these studies LCPs that distinguish the two main pathological hallmarks of AD, A-beta plaques and neurofibrillary tangles (NFTs). The LCPs, pFTAA, hxFTAA and hFTAA, give a distinct spectroscopic signature from A-beta or tau aggregates (**Figure 17**). Notably these LCPs were five to seven membered oligo-thiophenes containing two additional carboxylic moieties terminating the conjugated backbone. These data were published in Organic and Biomolecular Chemistry 2011.

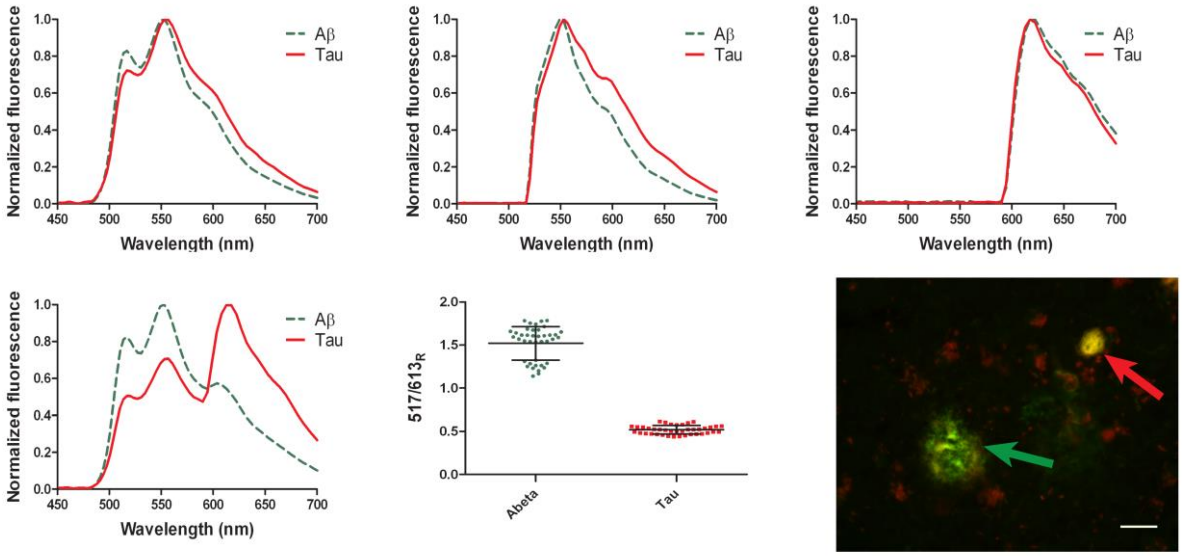


Figure 17: *Emission spectra and fluorescence images of p-FTAA bound to pathological hallmarks in AD human brain tissue. The emission spectra of the indicated LCO bound to Aβ plaques (green dashed line) or NFTs (red solid line) when excited at 405 nm (top left), 480 nm (top middle) or 546 nm (top right). Merged emission spectra (exc: 405 and 546 nm) of p-FTAA, bound to Aβ plaques (green solid line) or NFTs (red dashed line) (bottom left). Plot of the ratio of light intensity emitted at 517 and 613 nm (517/613R) from p-FTAA bound to Aβ plaques (green triangles) or NFTs (red squares) shown with SD (bottom middle). Fluorescence images of human AD brain sections stained with p-FTAA (bottom right). The images visualize typical pathological entities, Aβ plaques (green arrow) or NFTs (red arrow), from where the emission spectra were obtained. Scale bar represent 20 µm.*

Therapeutics

LCPs as pharmacophores in AD mouse models

One aim with LUPAS was to test the potency of LCPs for possible pharmacological effects in transgenic mice with AD pathology. We tested LCOs as possible pharmocophores *in vivo*

because pFTAA is known to target pre-fibrillar amyloid species, thereby possibly generating minor alterations in the molecular structure of amyloid fibrils that may interfere with amyloid fibril formation and their putative toxicity. It was demonstrated that p-FTAA (LCP 5001) is able to cross the blood brain barrier after intravenous (i.v.) and intraperitoneal (i.p.) injection and bind to cerebral amyloid plaques of *APPPS1* mice, an early onset mouse model of cerebral amyloidosis. Repetitive injections of p-FTAA resulted in a robust staining of cerebral amyloid plaques in *APPPS1* mice, and probes remained bound to plaques for up to 6 months. APPPS1 mice were treated for 12 weeks by weekly i.p. p-FTAA-injections and Aβ pathology and the general health was studied. Despite efficient binding of p-FTAA to cerebral amyloid, no significant p-FTAA dependent alteration in total brain amyloid burden, as judged by Congo Red staining and Aβ-immunohistochemistry was observed. These data indicate that persistent peripheral p-FTAA treatment neither causes obvious signs of toxicity, nor alters cerebral amyloid load in APPPS1 mice. To study whether continuous p-FTAA treatment beyond morphologically detectable alterations may modify amyloid toxicity on a functional level, long term potentiation and cognition in p-FTAA-treated APPPS1 mice will need to be assessed. This study was presented at the Foundation Ipsen conference in Paris February 2012.

S. Handrick, S. Prokop, S. Nystrom, J.J. Mason, N.R. Reitan, B.M. Wegenast-Braun, M. Lindgren, M. Jucker, K.P. Nilsson, P. Hammarstrom, F.L. Heppner Luminescent Conjugated Oligothiophenes (LCOs) as Pharmacophores in Alzheimer's Disease. Fondation Ipsen, Paris (2012)

Using LCP fluorescence as a follow up during treatment

For several years, the natural compound curcumin has been proposed to be a candidate for enhanced clearance of toxic Aβ amyloid. Herein LUPAS researchers studied the potency of feeding curcumin as a drug candidate to alleviate Aβ toxicity in transgenic Drosophila. The longevity as well as the locomotor activity of five different AD model genotypes, measured relative to a control line, showed up to 75% improved lifespan and activity for curcumin fed flies. In contrast to the majority of studies of curcumin effects on amyloid we did not observe any decrease in the amount of Aβ deposition following curcumin treatment. Importantly, conformation-dependent spectra from p-FTAA, LCP (5001) bound to Aβ deposits in different Drosophila genotypes over time, indicated accelerated pre-fibrillar to fibril conversion of Aβ(1-42) in curcumin treated flies. Our study shows that curcumin promotes amyloid fibril conversion by reducing the pre-fibrillar/oligomeric species of Aβ, resulting in a reduced neurotoxicity in Drosophila. Most importantly for LUPAS was that LCP fluorescence is an important tool for assessing conformational differences in treated versus untreated animals.

This study was published in PLoS One February 2012 Caesar I, Jonson M, Nilsson KP, Thor S, Hammarström P. Curcumin promotes A-beta fibrillation and reduces neurotoxicity in transgenic Drosophila. PLoS One. 2012;7(2):e31424. doi: 10.1371/journal.pone.0031424. This publication has been viewed on line >5600 times and was featured in >20 News articles worldwide.

Inflammatory modulation for new treatment strategies

Work within LUPAS also included assessment of alternative treatment strategies. The pathology of Alzheimer's disease has an inflammatory component that is characterized by upregulation of proinflammatory cytokines, particularly in response to amyloid-β (Aβ). The *APPPS1* Alzheimer's disease mouse model showed increased production of the common interleukin-12 (IL-12) and IL-23 subunit p40 by microglia. Genetic ablation of the IL-12/IL-23 signaling molecules by deficiency of p40 or its receptor complex, resulted in decreased cerebral amyloid load. In addition, peripheral administration of a neutralizing p40-specific antibody also resulted in a reduction of cerebral amyloid load in *APPPS1* mice. Furthermore, intracerebroventricular delivery of antibodies to p40 significantly reduced the concentration of soluble Aβ species and reversed cognitive deficits in aged *APPPS1* mice. The concentration of p40 was also increased in the cerebrospinal fluid of subjects with Alzheimer's disease, which suggests that inhibition of the IL-12/IL-23 pathway may attenuate Alzheimer's disease pathology and cognitive deficits. The paper "Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline" by Vom Berg J, Prokop S, Miller KR, Obst J, Kälin RE, Lopategui-Cabezas I, Wegner A, Mair F, Schipke CG, Peters O, Winter Y, Becher B, Heppner FL.. Nat Med. 2012 Dec;18(12):1812-9 was recently published. This paper was highlighted in Nature Reviews Neuroscience 2013, Nature Reviews Drug Discovery 2013, and Nature Reviews Immunology 2013, New England Journal of Medicine, 2013.

Follow up of these mouse models by LCP fluorescence should add another level of analysis not only limited to plaque load but also LCP spectroscopy can delineate conformational differences within plaque. Such studies have so far been limited by the studied age groups of 4 and 8 months. Large differences in LCP spectroscopic differences in APP/PS1 mice occur after the age of 12 months (unpublished results), hence the time window needs to be adjusted for such studies to be meaningful.

Prions

Prion diseases or transmissible spongiform encephalopathy (TSE) have received unprecedented public interest despite its rareness in the human population. The main reason for this interest was the epidemic of bovine spongiform encephalopathy (mad cow disease) some 20 years ago and its evident transmissibility from cattle to humans. Prions can reside silent for decades in an infected individual and pose a threat to the human population due to its efficiency in jumping species barriers and through blood transfusions and

transplants. Prion diseases are hence of outmost interest not only for basic research curiosity but rather it is imperative to possess sensitive detection and therapeutic strategies. The culprit protein in all prion diseases is called the prion protein. The prion protein (PrP) is most abundant in mammalian neurons but is ubiquitously expressed throughout various cells and tissues. The functional role of native PrP is not fully understood. PrP is associated with a number of different prionoses: sporadic, inherited, and acquired, all of which are invariably fatal. The common molecular pathognomonic marker for prionoses is the presence of severe vacuolation within the CNS rendering a sponge-like tissue. Concomitant with the presence of spongiosis is the presence of a conformational isoform of PrP, which has converted from a largely helical globular protein PrP^{C} that misfolds into an aggregation prone β-sheet conformation, PrP^{Sc}, which often assemble into protein deposits with conspicuous similarities to amyloid.

The concept of prions as a novel pathogenic entity composed solely of corrupt protein has been extensively debated in several important review articles by LUPAS researchers. LUPAS researchers recently published "Prion propagation, toxicity and degradation" by A. Aguzzi and J. Falsig in nature Neuroscience 2012. Nat Neurosci. 2012 Jun 26;15(7):936-9, to address this topic.

Development of prion assays

Lack of cytotoxicity in prion infected cell cultures has hampered mechanistic studies of prion-induced neurodegeneration. Recently, LUPAS researchers showed that prion-infected cultured organotypic cerebellar slices (COCS) experienced progressive spongiform neurodegeneration closely reproducing prion disease, with three different prion strains giving rise to three distinct patterns of prion protein deposition. Neurodegeneration did not occur when PrP was genetically removed from neurons, and a pharmacological screen indicated that neurodegeneration was abrogated by compounds known to antagonize prion replication. Prion infection of COCS and mice led to enhanced fodrin cleavage, suggesting the involvement of calpains or caspases in pathogenesis. Accordingly, neurotoxicity and fodrin cleavage were prevented by calpain inhibitors but not by caspase inhibitors, whereas prion replication proceeded unimpeded. The article was published in PLoS Pathogens in 2012: "Prion pathogenesis is faithfully reproduced in cerebellar organotypic slice cultures". Falsig J, Sonati T, Herrmann US, Saban D, Li B, Arroyo K, Ballmer B, Liberski PP, Aguzzi A. PLoS Pathog. 2012 Nov;8(11):e1002985.

Therapeutics of prion diseases

Prion diseases in rodents faithfully reproduce the human disease, in contrast to Alzheimer's disease in mice, which only show subtle pathophysiological phenotypes. This makes prion disease models a powerful assay for novel therapeutics that targets the culprit disease causing misfolding protein. LCPs interact with ordered protein aggregates and sensitively detect amyloids of many different proteins, suggesting that they may also possess antiprion properties. Eight different LCPs were analyzed with a variety of side chain and chain lengths for antiprion activity. A variety of anionic, cationic, and zwitterionic LCPs reduced the infectivity of prion-containing brain homogenates and of prion-infected cerebellar organotypic cultured slices (COCS) (**Figure 18**) and decreased the amount of scrapie isoform of PrP^{C} (PrP^{Sc}) oligomers that could be captured in an avidity assay. Paradoxically, treatment enhanced the resistance of PrP^{Sc} to proteolysis, triggered the compaction, and enhanced the resistance to proteolysis of recombinant mouse PrP(23–231) fibers. These results suggest that LCPs act as antiprion agents by transitioning PrP aggregates into structures with reduced frangibility. Moreover, ELISA on cerebellar organotypic cultured slices and *in vitro* conversion assays with mouse PrP(23–231) indicated that poly(thiophene-3-acetic acid) (LCP 10001) may additionally interfere with the generation of PrP^{Sc} by stabilizing the conformation of PrP^{C} or of a transition intermediate. Therefore, LCPs represent a novel class of antiprion agents whose mode of action appears to rely on hyperstabilization, rather than destabilization, of PrP^{Sc} deposits (**Figure 19**).

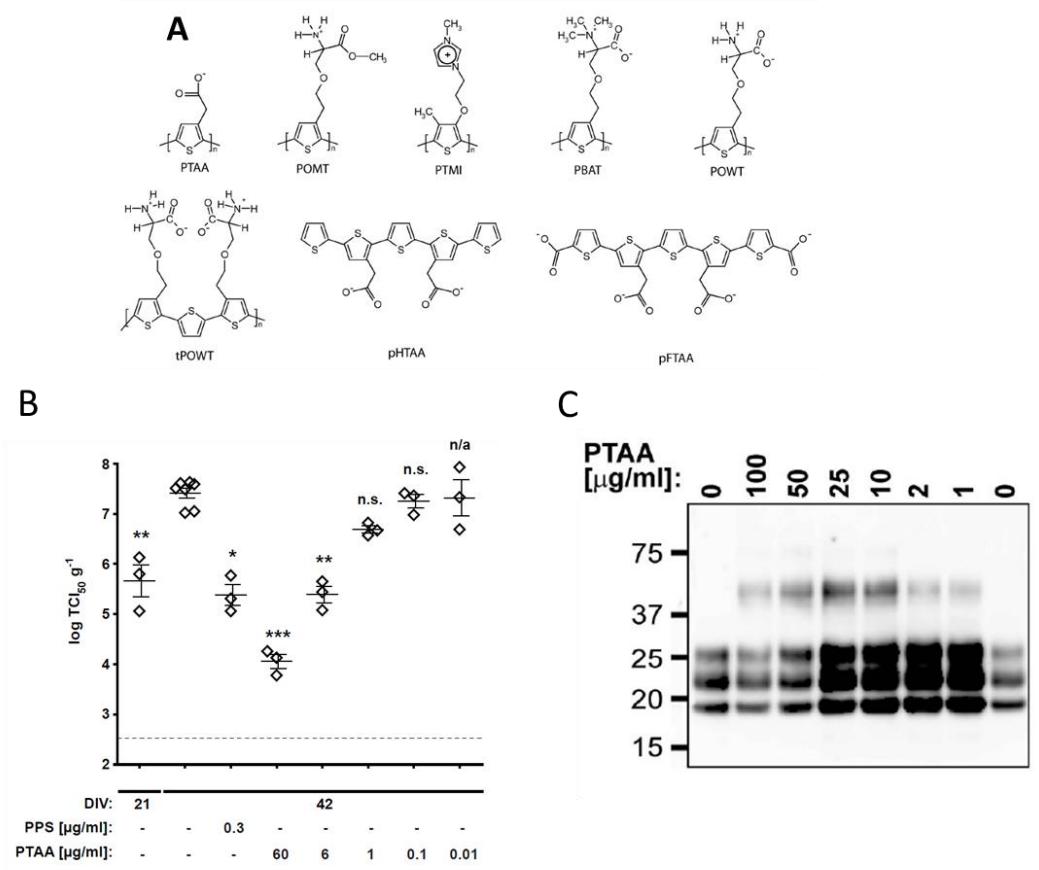


Figure 18: *LCPs as antiprion agents. A) The chemical structure of the 8 different LCPs of the study. B) Prion infectivity titers formed in oranotypic cerebellar slices as a function of treatment with PTAA (LCP 10001) or control compound PPS. C) Proteinase K resistance as a function of PTAA concentration, showing a window of concentrations where PK resistance increase. Figure adapted from Margalith et al., 2012, JBC.*

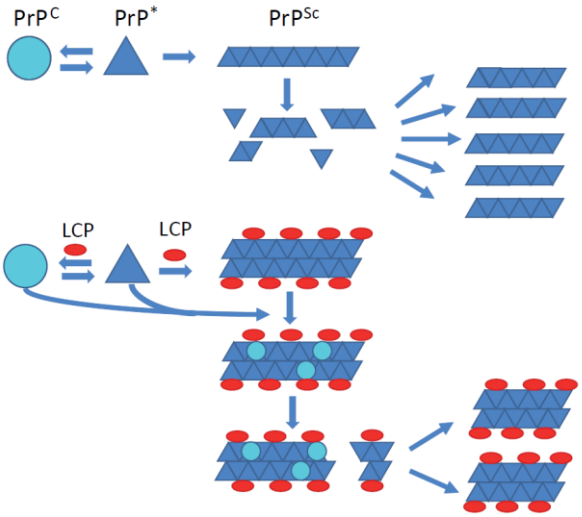


Figure 19: *Model for antiprion activity of LCPs.* Top: *in the prion model PrP^C is in a reversible thermodynamic equilibrium with PrP*, which further aggregates into amyloid fibrils, PrP^{Sc}. When the fibrils reach a critical length, the fibril becomes more fragile, and fragmentation occurs. The newly formed ends of the fibril fragments are new nucleation sites for further fibril propagation.* Bottom: *antiprion activity of the LCPs seems to be based on interactions with PrP^{Sc} aggregates, possibly increasing their compactness. LCP-coated fibrils further embed pre-existing prions and even PrP^C. The higher compactness of the PTAA-treated aggregates ultimately become hyperstabilized and cause less fragmentation into infectious particles necessary for further prion replication. Figure adapted from Margalith et al., 2012, JBC.*

Diagnostics of human prion diseases

From a diagnostic perspective, the LCPs/LCOs were also used to analyse post-mortem tissue sections from patients with neurodegenerative diseases. The fluorescence stains selectively bind to the protein deposits and expose these major pathological hallmarks of either AD or prion diseased brain. Prion protein deposits from Creutzfeldt-Jakob disease are notoriously complicated to visualize using conventional fluorescence dyes for amyloid such as Congo red and Thioflavin T. Several LCPs function well in this regard as was tested extensively on mouse material. To employ frozen material is especially important in this context. Within LUPAS this complicated procedure from a biosafety perspective was achieved. When this hurdle was overcome identification of PrP deposition in TSE brain can easily be done and distinguished due to the color emitted from the LCP/LCO bound to the different entities as shown in **Figure 20** for three distinct prion diseases. Hence, the LCP/LCO technique shows excellent promise for being implemented as a complementary technique in clinical diagnostics of TSEs.

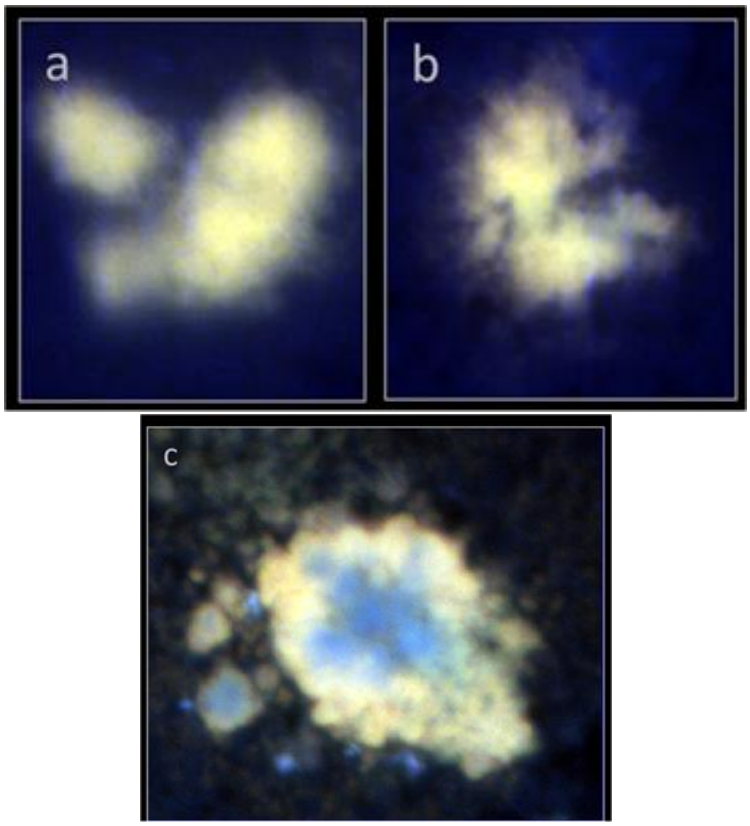


Figure 20: *LCP fluorescence from protein aggregates in frozen brain tissue from human patients with prion disease: a) Creutzfeldt-Jakob Disease (CJD)type I, b) CJD type II, c) Inherited prion disease Gerstmann-Straussler-Scheinker Disease (GSS).*

Potential impact including Socio-economic impact

The European population is aging. As a consequence age-related neurodegenerative diseases such as Alzheimer's disease are on a steady rise reaching epidemic proportions of serious concern to the afflicted patients and their relatives. These diseases put a heavy burden on society including a decade of profound dependence on caregivers culminating with care at nursing homes. If this epidemic is not sequestrated it is projected to threaten the core of the well-fare societies of all European Nations.

The most thorough data on the ongoing and forthcoming Alzheimer epidemic comes from the Alzheimer Association is the USA (www.alz.org). European national and transnational initiatives (Alzheimer Europe www.alzheimer-europe.org) are emerging initiatives to tackle the rising incidence of dementias. In 2010 the European Commission funded the Joint Programming Initiative on Alzheimer's disease and neurodegenerative diseases (JPND) with an EU contribution of close to EUR 2 million. From the Alzheimer Europe initiative it reads

that "*In 2005, the total direct and informal care costs of Alzheimer's disease and other dementias were estimated at €130billion in the EU27 (€21 000 per patient); 56% of these costs was informal care. The most common forms of dementia in the European Union are Alzheimer's disease (about 70% of cases), and vascular dementia (less than 30%*)". These are staggering figures and are projected to rise.

A patient is diagnosed with Alzheimer's disease mainly through the primary care where changes in short term memory and personality is based on cognitive tests. The cognitive tests are combined with MRI scans to rule out brain tumors or stroke as a primary cause for the symptoms. More developed centers allows for cerebrospinal fluid testing for biomarkers such as increased amounts of Tau and phosphorylated Tau and decreased amounts of soluble Aβ42. The culprit within these diseases is accumulated misfolded protein molecules, called amyloids of e.g. the mentioned biomarkers, which corrupt normal cellular functions and cause disease. Amyloids are self-perpetuating fibrillar states, which can hide for decades within affected individuals and replicate with immense efficiency, especially in the elderly. We, as a community, still stand short in delivering definite diagnosis or benign disease-modifying treatment or cure to any neurodegenerative disease. The only way to fight these diseases is long-term focused high quality research.

It is highly encouraging that the European Commission distributes increasing amounts of funding for tackling these diseases and hence work towards novel ideas on how to make disease modifying therapies and rapid diagnostics and follow up on treatments are ongoing in projects such as the described LUPAS project.

The LUPAS project is a testament to progress in cross-disciplinary research with a very impressive output of scientific impact. Over the years LUPAS researchers have been met with enthusiasm from the scientific community as feedback on published papers and conference presentations. LCP molecules have been sent to more than two dozen labs worldwide and a dozen collaborations have been initiated.

Final outcome & future perspectives

There is a tremendous need for quantitative diagnostic methods for early detection and evaluation of neurodegenerative disorders, such as Alzheimer's disease and prion diseases. The need is underlined by the recent development of proposed therapeutical interventions targeting disease, so called disease modifiers, including immune therapy. Herein, quantitative physical outcome measures are urgently needed in terms of amyloid pathology within living patients.

Within the brief 3 year time frame of LUPAS, the consortium developed important research tools for use in disease model systems (mouse models) in vivo and on histological ex vivo

samples from humans. The LUPAS project exemplifies that broad cross-disciplinary expertise within diverse subjects can realize unmet needs within biomedicine. The realistic prognosis is that it will take a few more years to validate this technology in the preclinical phase prior to exploiting it in the clinic and hence lead to:

- Improved diagnostics of Alzheimer´s disease
- Improved diagnostics of prion disease
- New avenues for therapeutics in Alzheimer´s disease
- New avenues for therapeutics in prion disease
- Improved diagnostics of systemic amyloidosis

The LUPAS consortium strives for continuing towards these goals beyond the project time frame pending future generous support from various stakeholders such as industry, academia, patients and politicians.

Economic impact of biomedical imaging

The value of LCP molecules becomes tangible and visible in the light of the various applications and proof-of-concept that has emerged as results from the LUPAS project. The potential business case needs to be developed and discussed from a strategic point of view. This brief market analysis is a first step towards understanding the market and market players. A market analysis should preferably cover the full potential of the LUPAS project outcome. That is all kinds of imaging applications from basic research to clinical applications as well as molecular diagnostics and therapeutic use. Herein we decided to focus on the imaging market. Today medical imaging technologies are widely used in clinical diagnosis to guide therapeutic and surgical intervention and to monitor disease progression, recurrence and treatment response and to improve surgical navigation. The clinical market is a multi billion market industry. The markets for PET tracers and MRI contrast agents, which are the most relevant markets in the context of LUPAS was estimated to USD 2.3 billion in 2011. The major players in the market are companies like GE, Bracco, Bayer and Guerbet. The market for radiopharmaceuticals and molecular imaging is expected to experience strong growth. Nuclear medicine, including SPECT and PET, is revolutionizing how diseases are treated, such as Alzheimer's disease. The FDA in early 2012 approved Amyvid (Florbetapir F 18 Injection) (Lilly/AvidRadiopharmaceuticals) for PET imaging of the brain in adults who are being evaluated for Alzheimer's Disease (AD) and other causes of cognitive decline. Neoprobe has licensed from AstraZeneca the worldwide rights to an imaging agent called AZD4694, which is a Flourine-18 labeled radiotracer intended for use in the diagnosis of Alzheimer's disease. The deal calls for Neoprobe to make a $5 million up-front payment to AstraZeneca. Terms of the deal also include up to $6.5 million in payment, contingent upon the achievement of clinical and regulatory milestones, and another payment of up to $11 million. The LUPAS

project has shown that LCPs have a clear potential in imaging and for clinical use the first choice is a PET imaging agent. The combination of imaging and spinal fluid diagnostics is a novel possibility, which needs to be analysed further. Obviously, the LUPAS project has not at this stage performed a study for proof-of-concept for the purpose, however the data accumulated so far in combination with the size and growth of the target market is intriguing. Perhaps most importantly from a strategic point of view, is to define the competitive edge of the LCPs in this case. It will probably be more interesting to present a reagent that can move diagnostics of amyloid plaques one step further than the agents already approved for clinical use. It is of great importance to stress the benefit from a healthcare and patient perspective in using a LCP derived agent in comparison with the existing imaging methods. If there is a few distinct answers to that question, like for instance the possibility to image conformational differences in the plaques and correlate that output data to guidance for treatment, the probability to be able to get an approval and a significant market share increases rapidly. The preclinical contrast agent market is valuated to $200 million (2010) and it is expected to grow with a double digit on an annual basis the next couple of years. It is being dominated by optical imaging reagents ($70 million) followed by PET/SPECT radionucleotides ($60 million) and MRI ($40 million). It is important to notice that the preclinical market utilizes contrast agents approved for clinical use as well as novel agents. The preclinical arena is therefore an important test for novel agents. Multimodal nanoparticles are one example as well as novel fluorescent biomarkers and PET/SPECT tracers. It is obvious that the LCPs recognition of amyloid and prion based deposits in combination with the fluorescent signature, radionucleotide labelling and the conjugation of LCPs to nanostructure open novel imaging possibilities. Optical imaging is already proven very well within the LUPAS consortium and the only limitation market wise may be the need for adequate animal models and technical skills that are required to perform optical imaging within the brain. An MRI contrast agent that can cross the blood-brain barrier and utilize LCP specificity would gain great interest and a field of research that is interesting to continue after the LUPAS project as such. In any preclinical imaging study the need for an orthogonal method to confirm your images is needed. In this case the LCPs inherent fluorescent properties makes it ideal for post mortem tissue microscopy to confirm the findings from the in vivo imaging studies. Taken together these features are very promising in the perspective of commercializing a multimodal imaging toolbox for the preclinical arena for applications within Alzheimers and Prion deseases. The market drivers and instrumentation available give on hand that it is interesting to market LCPs on the preclinical market.

For companies like Genovis and Applied Spectral Imaging, both partners within the LUPAS project, it may be valuable to further investigate the preclinical and tissue imaging markets in order to extend their respective business. To provide unique biomarkers and contrast agents in the field of Alzheimers research will add a competitive edge and a substantial

market potential for reasonable investment. There are a few challenges in addition to performing a more in-depth competitive edge analysis. The most important hurdles to overcome initially is to set up a production source for the LCPs, move around technology barriers by providing application notes and reference studies, show that the LCPs are non-toxic and prove multimodality.

Project website address: http://www.lupas-amyloid.eu/