Detection and Remodeling of Toxic Amyloid Aggregates by Novel Conjugated Polyelectrolytes

Adeline Marianne Fanni
University of New Mexico

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Detection and Remodeling of Toxic Amyloid Aggregates by Novel
Conjugated Polyelectrolytes

BY

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DISSERTATION
Submitted in Partial Fulfillment of the
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Dedication

I would like to dedicate this work to my parents who guided me throughout the years. They taught me to never give up on reaching my dreams. Their love and support made me who I am today. This dissertation is an accomplishment that I would not have been able to do without you.

I would also like to dedicate this to my boyfriend Joseph Dumont who is the reason why I started a Ph.D. in a new county. Thank you for your patience and for supporting me throughout these years.
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Abstract

The abnormal misfolding and aggregation of intrinsically disordered proteins into ordered fibrillar amyloid aggregates is recognized as a common pathological event in neurodegenerative disorders (ND) such as Alzheimer’s and Parkinson’s diseases. The key role of fibrillar and pre-fibrillar aggregates in ND makes them ideal biomarkers to better understand disease progression but also for early disease detection and therapeutic intervention. The work described herein was conducted to gain a better understanding of amyloid interaction to lipid membrane and its role in peptide toxicity. This research was also focused on evaluating the fluorescent probe, oligomeric \( p \)-phenylene ethynylene (OPE), as an amyloid sensor detecting disease-relevant pre-fibrillar and fibrillar aggregates both \textit{in vitro} and in biological samples. Finally, OPE’s capability to selectively photo-oxidize amyloid fibrils to ensure their disassembly and clearance was investigated. Taken together, this study revealed the potential of OPE as a theranostic agent for neurodegenerative disorders.
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Chapter 1: Introduction

1.1 Neurodegenerative Disorders

Neurodegenerative disorders are a group of diseases characterized by a progressive deterioration of the central nervous system causing memory loss and cognitive impairments, such as Alzheimer’s and Parkinson’s diseases\(^1\). In 2015, 46.8 million people were affected by these disorders worldwide, and this number is expected to double over the next 20 years due to the rise of life expectancy\(^2\). These diseases are currently diagnosed through clinical symptoms analysis and are confirmed postmortem based on neuropathological biomarkers\(^3\).

To this day, these disorders cannot be cured, and the predicated cost of patients care in the United States is one of the highest ($259 billion for dementia care vs $157 billion for cancer care)\(^4,5\). The cause of neurodegeneration is still unclear, but a common pathological hallmark has been reported among these different disorders: accumulation of amyloid aggregates\(^6-8\).

Amyloid aggregates can be made of a large array of proteins such as amyloid β (Aβ) and tau proteins that form amyloid plaques and neurofibrillary tangles in Alzheimer’s disease, or α-synuclein proteins composing the Lewy bodies in Parkinson’s diseases\(^9\). Despite the high polymorphism of these aggregates, they all share a common secondary structure rich in β-sheet.

1.2 Amyloid Aggregation Mechanism

Amyloid aggregation is a complex process which takes place through a nucleation-dependent polymerization mechanism\(^10\). Two pathways have been described: primary and secondary nucleation. During the primary nucleation, the native protein misfolds in an intermediate prone to aggregation which will form oligomers and protofibrils, also called pre-fibrillar aggregates.
(Figure 1-1A). These pre-fibrillar aggregates act as seeds that grow into stable fibrillar aggregates rich in β-sheet. The secondary nucleation consists in the formation of aggregates through fibril fragmentation or “surface catalyzed nucleation” where fibrils are used as scaffold by monomeric proteins to produce aggregates (Figure 1-1B).

![Diagram](image)

**Figure 1-1:** Nucleation dependent aggregation (A) primary nucleation (B) secondary nucleation mechanism.

Protein aggregation is believed to start decades before the onset of clinical symptoms. Additionally, there is evidence that pre-fibrillar aggregates are more labile and neurotoxic than amyloid plaques. It is believed that they exert their toxicity through several mechanisms such as cell membrane disruption leading to cellular homeostasis disruption and cell apoptosis, or by inducing local inflammation upon binding to membrane or by...
interacting with specific membrane-receptors\textsuperscript{23, 24}. Despite their lowest toxicity, amyloid fibrils also play a role in neurodegeneration through several mechanisms such as impairing axonal transport\textsuperscript{25, 26}. The key role of the amyloid aggregates in neurodegenerative disorders make these fibrillar and pre-fibrillar aggregates ideal biomarkers for early disease detection and therapeutic intervention.

1.3 Membrane-Mediated Toxicity, Fibrillation and Transmissibility of Amyloid Proteins

The neurotoxicity of amyloid aggregates is still poorly understood; however, many have shown that amyloid proteins capability to interact with the neuronal cell membrane, more particularly with anionic lipids, might play a central role\textsuperscript{27, 28}. This interaction is believed to play a key role in protein toxicity,\textsuperscript{27, 28} protein fibrillation\textsuperscript{29-33} and disease spatial progression\textsuperscript{34}.

Many studies have shown that upon interaction to cell membrane, amyloid proteins destabilize the lipid packing through three main mechanisms: (1) “carpeting-effect mechanism”, (2) “pore-formation mechanism” and (3) “detergent-like mechanism”\textsuperscript{35} (Figure 1-2). Through the “carpeting-effect mechanism” the amyloid protein blankets the lipid membrane causing membrane thinning. The carpeting effect also results in an asymmetric pressure between the two lipid layers and causes the generation of membrane curvature. “Pore formation mechanism” consists in the permeation of the membrane by the formation of ion-channel-like structures by the amyloid proteins. Membrane disruption can also be caused by a “detergent-like mechanism” where the proteins form a micelle-like structure with the lipid membrane. Overall, this membrane destabilization causes a loss of membrane integrity leading to cell apoptosis.
Figure 1-2: Schematic of the membrane disruption mechanism associated with amyloid protein interaction to lipid membrane: carpeting-effect, pore-formation and detergent-effect. Modified from Williams and Serpell, 2011.\textsuperscript{35}

There is evidence that upon interaction to the lipid membrane, amyloid proteins such as Tau\textsuperscript{29, 30}, Aβ\textsuperscript{31, 36} and α-synuclein\textsuperscript{32, 33} also undergo fast fibrillation. This membrane-mediated fibrillation might be caused by (1) partial folding of the protein upon interaction to the hydrophobic lipid membrane, (2) change in the orientation of the protein and (3) increase in the local protein concentration\textsuperscript{37}. Membrane-mediated fibrillation seems to be affected by many parameters such as the surface chemistry, the surface roughness or membrane curvature\textsuperscript{36, 38}.

Membrane interaction is also believed to play a role in the cell-to-cell transmissibility of amyloid proteins, which is responsible for the predictable spatial progression of various neurodegenerative disorders\textsuperscript{49}. In fact, cell internalization of tau aggregates is believe to be involved in transcellular propagation of the disease by inducing fibrillization of intracellular tau\textsuperscript{34}. These observations involved the presence of cellular pathways mediating the release and uptake of protein aggregates from the neuronal cells that are still poorly understood.

Overall, the molecular changes associated with protein-lipids membrane interaction implicated in membrane destabilization, protein fibrillation and cell-to-cell transmissibility are still elusive. A better comprehension of these mechanism could guide the development of novel therapeutic strategies.
1.4 Amyloid Aggregates as Biomarkers

The deposition of amyloid aggregates in the brain has been correlated with cognitive impairments in patient suffering of neurodegenerative disorders, which make them ideal biomarkers for both early diagnosis and therapeutic intervention. Recently, physicians have also reported a correlation between neurodegeneration and increase in amyloid oligomers in cerebrospinal fluid (CSF) that reach up to 1 pg/mL in AD patients\textsuperscript{40, 41}.

The scientific community is currently developing and evaluating new probes for the selective detection and imaging of amyloid aggregates composed of a large array of proteins. Histological stains such as thioflavin T (ThT) and congo red (CR) are being used to detect a wide range of protein amyloids\textsuperscript{42, 43}. Both dyes are believed to arrange themselves along the long axis of the fibrils and intercalate perpendicularly to the β-strands in the β-sheets (Figure 1-3). Once bonded to fibrils, ThT displays a fluorescence red shift accompanied by a large fluorescence enhancement while CR shows a change in light polarization leading to apple-green birefringence\textsuperscript{43-45}. Both dyes present several limitations. CR lacks sensitivity and has been shown to interact non-selectively with native proteins with various secondary structures, which could lead to false-positive results\textsuperscript{43, 46}. ThT is more specific than CR for the detection of amyloid fibrils. However, ThT binding is affected by fibrils morphology such as the flatness of the β-sheet,\textsuperscript{47} which explains ThT lack of detection of heavily twisted β-sheet-rich fibrils made of several proteins such as chymotrypsin or bovine IgG\textsuperscript{43, 48, 49}. Overall, the influence of fibrils morphology on ThT sensing properties makes this probe ineffective for the detection of a wide range of amyloid proteins with high polymorphism.
Figure 1-3: The common structure of fibrils and a structural rationale for fibril-ThT interactions (Modified from Biancalana and Koide, 2010). A. Amyloid fibrils are made of cross-β structure that forms layers of laminated β-sheets. B. ThT interaction to amyloid fibrils is proposed to follow a “channel” model where ThT binds along the surface side-chain grooves running parallel to the long axis of the β-sheet. ThT is represented by the brown arrow.

Radiopharmaceutical probes are used to image protein amyloids in vivo through different tomographic imaging techniques such as Positron Emission Tomography (PET). The main PET probes currently used for the detection of amyloid plaques are the non-FDA approved 11C-Pittsburgh Compound-B used in clinical research and three FDA-approved probes used in clinical trials: 18F-flutemetamol (Vizamyl™), 18F-florbetapir (Amyvid™) and 18F-florbetaben (NeuraCeq™). Despite the high selectivity and specificity of these probes toward amyloid plaques found in AD, development of efficient probes to detect α-synuclein accumulating in Lewy bodies in PD, or tau proteins accumulating in neurofibrillary tangles...
in AD$^{56, 57}$, are still in its infancy. Additionally, these PET probes are inadequate at detecting oligomers and protofibrils, the putative toxic species$^{56}$. These limitations call for the need to develop new sensors to detect early aggregates and mature fibrils made of different amyloid proteins such as Aβ, tau and α-synuclein that are involved in neurodegenerative diseases. These new probes should display high selectivity toward the amyloid conformation, low limit of detection and low interference by other biomolecules such as albumin or collagen present in biological fluids or in the brain. These new probes would assure a better understanding of the disease, guarantee a more reliable diagnosis, and be a valuable tool for evaluating new treatments.

1.5 Therapeutic Interventions

The anticipated increase of patients with neurodegenerative disorders in the next 15 years and the cost associated to patient care give rise to the urgent need to develop an efficient therapy. Protein aggregation plays a key role in neurodegenerative disorders and is an ideal target for therapeutic intervention$^{58-60}$. The main therapeutic strategies targeting amyloid proteins can be organized in three groups$^{61}$:

1. **Inhibition of amyloid aggregation and fibrillization**$^{62}$. Several compounds have already been studied for their capability to inhibit the nucleation-dependent aggregation such as flavonoids (epigallocatechin gallate$^{63}$), surfactants$^{64}$, small peptides$^{65, 66}$ or switchable photo-oxygenizers$^{67-69}$. This last class of compounds has recently been described for their capability to reduce fibril production and lower aggregate neurotoxicity by oxidizing amyloid proteins.
2. **Amyloid remodeling into non-toxic oligomers.** Small molecules such as curcumin\textsuperscript{70} or keampferol-3-O-rhamnoside\textsuperscript{71} have been shown to modify the amyloid aggregates into non-toxic aggregates without inhibiting fibrils production.

3. **Amyloid degradation and clearance** which consists in destabilizing and/or disassembling pre-fibrillar and fibrillar aggregates. Several strategies have been investigated to induce amyloid degradation such as the use of proteases\textsuperscript{60, 72, 73} or amyloid photo-oxidizers such as methylene blue (MB)\textsuperscript{67-69}.

The evaluation of new compounds targeting the amyloid cascade would assure a better understanding of the amyloid hypothesis and could initiate the development of new strategies to treat neurodegenerative diseases.

### 1.6 Amyloid Degradation and Clearance by Photodynamic Therapy

Photodynamic therapy (PTD) is commonly used in dermatology and oncology for the treatment of various tumors and nonmalignant diseases\textsuperscript{74-76}. This strategy consists in the administration of a photosensitizer (PS) followed by its illumination at a specific wavelength. Upon irradiation, the PS reaches an excited singlet state (Sn) which will decay back to the ground state through quenching or by emitting fluorescence (Figure 1-4). The PS can also undergo electron spinning leading to the generation of a triplet excited state (T1). From the triplet state, the PS will form free radicals through electron transfer or will generate singlet oxygen species through the type I and II reactions, respectively (Figure 1-4)\textsuperscript{76, 77}. An efficient PS should accumulate in the targeted area, it should be light-controllable, it should have high quantum yields for the generation of singlet oxygen and also present low off-target reaction\textsuperscript{74}. 

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**Figure 1-4:** Modified Jablonski Diagram Describing the Photosensitization Type I and Type II Mechanisms. After absorption of a photon, the photosensitizer is activated from its ground singlet electronic state \( S_0 \) to an excited singlet state \( S_n \). Through internal conversion and vibrational relaxation, the singlet state \( S_1 \) is reached and can return to \( S_0 \) through quenching/non-radiative relaxation or fluorescence. \( S_1 \) energy can also be converted in an exited triplet state through intersystem crossing. \( T_1 \) can return to ground state through phosphorescence or can transfer its energy to a biological molecule (R) to form free radicals (Type I reaction) or to a triplet oxygen \( (^3O_2) \) to form singlet oxygen \( ^1O_2 \) (type II reaction) which will cause oxidative damage.

PDT has recently been investigated to photo-oxidize amyloid proteins implicated in neurodegenerative disorders. Several PS such as riboflavin\(^{67} \), rose bengal\(^{78} \), water-soluble porphyrin molecules\(^{79} \), flavin-based catalysts\(^{67, 68} \) or methylene blue (MB)\(^{69} \), were evaluated. They were shown to either photo-oxidize A\( \beta \) monomers and inhibit their aggregation\(^{67, 78, 79} \), or to photo-oxidize amyloid fibrils causing their disassembly into shorter structures characterized by lower cell toxicity\(^{68} \). PS currently evaluated for the oxidation of amyloid proteins such as MB, present one main drawback: low selectivity toward the toxic amyloid conformation. As a consequence, the native protein playing important physiological functions might be oxidized as well as other biomolecules such as protein, lipid and nucleic acids\(^{80} \) which could cause neuronal death. The off-target oxidation of current PS highlights the need to develop new photosensitizers highly selective toward the toxic amyloid aggregates.
1.7 Oligomeric \( p \)-Phenylene Ethynylenes – Amyloid Aggregates Detection

Oligomeric \( p \)-phenylene ethynylenes (OPEs) is a novel class of water-soluble fluorescent sensors which are highly versatile in terms of size (1, 2 or 3 repeat units) and charge (positively or negatively charged) ([Figure 1-5])\(^{81,82}\).

![Figure 1-5: OPEs structure\(^{81}\)](image)

OPEs are characterized by minimal background fluorescence due to ethyl ester termini quenching by water molecules\(^{83}\). Upon binding to hydrophobic scaffolds, OPEs display a large fluorescence enhancement caused by (1) backbone planarized, (2) hydrophobic unquenching and (3) formation of super-luminescent complexes mainly made of J-dimers\(^{81,82,84}\). J-dimers spectral signatures are summarized in [Figure 1-6]\(^{85}\). A large library containing 18 different phenylene ethynylenes-based oligomers were screened for the selective detection of the fibrillar conformation of two model proteins oppositely charged at neutral pH, the positively charged hen egg white lysozyme (HEWL) and negatively charged bovine insulin (BINS)\(^{81,82}\). The anionic OPE\(_1\)^{2-} made of one repeat unit and two side chains terminated by a sulfonate group (OPE\(_1\)- on [Figure 1-5]), was found to selectively detect both BINS and HEWL fibrils over their monomeric counterparts. The cationic OPE\(_2\)^{4+} made of two repeat units and four side chains terminated by a quaternary amine group (OPE\(_2\)+ on [Figure 1-5]), displayed stronger fluorescence enhancement in the presence of HELW fibrils, but did not detect selectively BINS.
fibrils likely due to electrostatic interactions between the positively charged OPE and the negatively charged monomeric protein.

The selective binding of OPE with the fibrillar conformation of two model proteins makes this sensor highly promising for the detection of fibrillar and pre-fibrillar aggregates composed of disease-relevant proteins.

**Figure 1-6:** Schematic representation of H and J-aggregates and the associated changes in the absorption (blue) and emission (red) spectra from cyanine dye monomers. H aggregates are produced from dye parallel stacking. They are characterized by a hypsochromic shift (a shift in the absorption to a shorter wavelength) and usually present low to no fluorescence. J-aggregates are made of scattered dye aggregates. They are characterized by a bathochromic shift (a shift in the absorption to a longer wavelength), a sharper emission peak, and a small Stokes shift (the difference in wavelength between positions of the peak of absorption and emission spectra).

1.8 Oligomeric p-Phenylene Ethynylenes – Photosensitizing Activity

The photosensitizing activity of OPE$_{12}$ was recently evaluated in the presence of a positively charged detergent cetyltrimethylammonium bromide (CTAB). Upon binding to CTAB, OPE was shown to display fluorescence turn-on which can be used for optical detection and to sensitize local singlet oxygen species through the generation of triplet state (**Figure 1-7**). This
photosensitization activity was found to be switchable with light illumination and through selective binding to a scaffold.

**Figure 1-7:** Schematic of detergent-induced self-assembly (DISA) model of OPE for optical detection and selective sensitization of singlet oxygen species.\textsuperscript{87}

OPEs switchable photosensitization of singlet oxygen could be used to selectively photooxidize hydrophobic scaffolds such as amyloid aggregates. Taken together, the selective turn-on fluorescence of OPE upon binding to the amyloid conformation of model proteins and its photosensitizing activity make OPE highly promising as a theranostic agent for early detection of amyloid aggregates and treatment of neurodegenerative diseases.
Chapter 2: Specific Aims and Overview

The key role of amyloid aggregates in neurodegenerative disorders make them ideal target to better understand disease progression but also for early disease detection and therapeutic intervention. The lack of information on amyloid toxicity mechanism and the lack of sensing probes guided the research described in this dissertation which present two main focuses:

1. First, characterizing the molecular-level changes associated with amyloid protein insertion into lipid membrane which is believed to play a key role in amyloid toxicity and fibrillation.

2. Secondly, evaluating OPE for the selective sensing and oxidation of toxic pre-fibrillar and fibrillar aggregates made of disease-relevant proteins which will illustrate the potential of this fluorescence sensor as a theranostic agent.

This research was organized in five specific aims:

1. Characterize the membrane mediated toxicity and fibrillization of Tau PHF6. PHF6 is the hexapeptide $^{306}$VQIVYK$^{311}$ located in the microtubule binding domain of Tau protein. PHF6 is believed to form the main core of Tau paired helical filament (PHF) and to play a key role in the interaction of tau protein with lipid membrane. To gain a better understanding of the membrane-mediated fibrillation of Tau protein, we characterized the molecular-level changes associated with the insertion of PHF6 into a negatively charged lipid membrane. In this study, two PHF6 isoforms were evaluated: the non-acetylated NH$_3^+$-PHF6 which present a positive net charge at pH 6 and the N-acetylated PHF6 (Ac-PHF6) which is neutrally charged at pH 6. By using these isoforms, we investigated the influence of the charge on peptide fibrillation potency and membrane interaction. First, peptide neurotoxicity and membrane permeation
activity were analyzed by MTS and Live/Dead assays, respectively. Then, PHF6 insertion into lipid membrane and the associated structural changes were characterized by *in situ* synchrotron X-ray reflectivity (XR) and grazing incidence X-ray diffraction (GIXD) on a lipid monolayer membrane at the air/water interface. Finally, PHF6 membrane-mediated fimbillation was examined in the presence of large unilamellar vesicle by using a combination of characterization methods including circular dichroism (CD) spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM) imaging. Thanks to this study, we gained a better understanding of tau’s membrane-mediated fimbillation and its role in neurodegenerative disorders.

2. **Evaluate OPEs selectivity and sensitivity for detecting fibrillar and pre-fibrillar aggregates made of disease-relevant proteins.** Previous studies have shown that OPEs interact selectively with the amyloid conformation of model proteins. In this study, we further evaluated OPE amyloid sensing properties through the detection of fibrillar and pre-fibrillar aggregates made of three different disease-related proteins, Aβ40, Aβ42 and α-synuclein. The binding efficiency of OPE$_1^{2+}$ and OPE$_2^{4+}$ were characterized through fluorescence measurement and by calculating the amyloid detection factor (ADF), the limit of detection, and the dissociation constant. We also compared OPE sensing properties to a well-known sensor, thioflavin T (ThT). This study contributed to a better understanding of OPE as an amyloid probe and more importantly we showed the potential of OPE for the detection of the most toxic species, pre-fibrillar aggregates. This research was published in 2019 in ACS Chemical Neuroscience titled “High Selectivity and Sensitivity of Oligomeric *p*-Phenylene
Ethynylenes for Detecting Fibrillar and Pre-Fibrillar Amyloid Protein Aggregates” (DOI - 10.1021/acschemneuro.8b00719). In addition to the work presented herein, our group is putting more effort to evaluate OPE as an amyloid probe both ex vivo and in vivo. I have contributed to the investigation of OPE for the sensing of neurofibrillary tangles in brain sections prepared from both human and mouse tauopathy model (rTg4510). We found that OPE$_{1^2}$ was a superior ex vivo sensor as it co-localized with the phosphorylated-tau at pThr231 (reactive to AT180 antibody) and detected neurofibrillary tangles at a lower concentration compared to ThT (1.5 mM for ThT vs 5 µM for OPE). This research is still on-going and will be completed by the graduate student Florencia Monge.

3. Characterize OPE as an amyloid dye in terms of background fluorescence, effect on amyloid aggregation and cell toxicity. To further characterize OPE as an amyloid dye, we first determined the optimal OPE$_{1^2}$ concentration for amyloid sensing by analyzing OPE background fluorescence at various concentration. We also evaluated the effect of OPE on amyloid aggregation when co-incubated with Aβ40 at different molar ratio OPE to protein (1:2, 1:5 and 1:10). Finally, we investigated OPE neurotoxicity. This study revealed that OPE does not alter the amyloid aggregation during co-incubation with the amyloid peptide and more importantly that OPE maintains its sensing properties. These results combined with the selective sensing of early pre-fibrillar aggregates, open the door to future applications such as the characterization of amyloid aggregation kinetic through real-time fluorescence microscopy.

4. Evaluation of oligomeric $p$-phenylene ethynylenes sensing properties in a complex biological sample, cerebrospinal fluid (CSF). Increase in amyloid oligomer
concentration in CSF has been correlated with cognitive impairment in AD\textsuperscript{88, 89}. A probe selectively detecting amyloid aggregates in CSF could be used to ensure early disease diagnosis. Such probe needs to be highly selective toward the aggregated conformation of the protein and its sensing properties should not be altered by the presence of other biological molecules such as glucose, salt and non-amylogenic proteins. To evaluate OPE sensing of amyloid aggregates in CSF, we first evaluated OPE\textsubscript{1}\textsuperscript{2-} and OPE\textsubscript{2}\textsuperscript{4+} background fluorescence in this complex environment. Then, OPEs sensing properties were investigated in healthy CSF spiked with amyloid aggregates at 21 µg/mL. We also investigated OPE detection of amyloid aggregates in CSF isolated from patients with dementia through the protein misfolding cyclic amplification (PMCA) assay. This assay consisted in using the seeding potency of the amyloid aggregates present in dementia CSF. For this assay, CSF samples were spiked with monomeric Aβ\textsubscript{40} which is expected to form fibrils in the presence of seed oligomers. Overall, this study contributed to a better understanding of OPEs sensing properties in a complex, disease-relevant biological sample and helped us investigate OPE as a diagnostic tool through the detection of amyloid aggregates in CSF.

5. **Evaluate the photosensitizing activity of OPE on amyloid fibrils.** Photo-oxidation of amyloid aggregates is a novel strategy currently investigated to inhibit protein aggregation and to assure fibrils disassembly\textsuperscript{67-69}. The challenge of such strategy is to develop a controllable photo-oxidizer selectively targeting amyloid aggregates. The selective binding of OPE and its photosensitizing activity were combined to develop a new switchable photo-oxygenation system. In this project, we evaluated OPE as an amyloid photo-oxidizer by monitoring Aβ fibrils oxidation by dot blot and mass
spectrometry. The consequences of fibrils photo-oxidation in terms of fibrils disassembly and fibrils toxicity were also characterized by TEM imaging and cell proliferation assay, respectively. The characterization of OPE as a controllable and selective amyloid photo-oxidation could contribute to the development of a novel generation of theranostic (‘therapeutic’+’diagnostic’) molecules for the detection and treatment of neurodegenerative diseases.
Chapter 3: Membrane-Mediated Fibrillation and Toxicity of the Tau Hexapeptide PHF6

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3.1 Abstract

The aggregation of the tau protein into neurofibrillary tangles is believed to correlate with cognitive decline in several neurodegenerative disorders including Alzheimer’s disease. Recent studies suggest that tau interaction with the cell membrane could serve as a toxicity pathway and also enhance fibrillation into paired helical filaments (PHFs). Conformational changes associated with tau-membranes interactions are poorly understood and their characterization could lead to a better understanding of tau pathogenicity. In this study, we investigated the molecular level structural changes associated with the six amino acid PHF6 peptide interaction with lipid membranes and characterized their effects on membrane stability and peptide fibrillation. Two forms of PHF6 were used, the aggregation prone PHF6 with N-terminal acetylation (Ac-PHF6) and the non-aggregation prone PHF6 with a standard N-terminus (NH₃⁺-PHF6). Both PHF6 peptides were found to be neurotoxic and exhibited similar membrane-mediated changes which consist of (1) favorable interaction with anionic membranes, (2) membrane destabilization through lipid extraction and (3) membrane-mediated fibrillation. The rate at which these changes occurred is the main difference observed between the two peptides. NH₃⁺-PHF6 shows slow membrane-mediated fibrillation after six days of incubation, while Ac-PHF6 adopted a β-sheet conformation at the surface of the membrane within hours. Ac-PHF6 interactions with the membrane were also accompanied by membrane invagination and rapid membrane destabilization. Overall, our results show that membrane interactions could play a critical role in tau toxicity and fibrillation and its characterization is important for significant advancement in the development of novel therapeutic strategies.
3.2 Introduction

The misfolding and aggregation of the microtubule-associated protein tau into neurofibrillary tangles (NFTs) is implicated in the development of Alzheimer’s disease (AD) and other tauopathies such as Down’s syndrome and frontotemporal dementia. The tau protein is composed of a projection domain made of acidic and proline-rich regions and a microtubule binding (MTB) domain containing up to four repeat units made of tubulin binding motifs (Figure 3-1). The physiological function of tau is to stabilize microtubules in order to ensure cytoskeletal organization and cellular trafficking. Tau’s function is modulated by post-translational modifications such as phosphorylation, which controls its affinity to the microtubules. In AD, tau is hyperphosphorylated which leads to the abnormal accumulation of unattached tau and promotes its self-aggregation into insoluble paired helical filaments (PHFs). The deposition of NFTs was found to correlate with cognitive decline and neurodegeneration, and is involved in the rapid transcellular propagation of the disease.

MTB domains play major roles in both tau’s physiological function and aggregation. Indeed, two hexapeptide segments, VQIINK (PHF6) and VQIVYK (PHF6) located in the second and third repeat units of the MTB domain, respectively, form the core of tau fibrils. Both hexapeptides form stable parallel-β-sheet twisted filaments similar to the β-sheet structures found in tau-oligomers and NFTs, which make them relevant models to investigate tau aggregation and toxicity pathways.

Tau’s toxicity mechanism is not well understood. However, recent studies indicate that tau binding to the lipid membrane disrupts cell membrane homeostasis and cause cell apoptosis. There are several hypothesized mechanisms by which amyloid proteins interact with and destabilize lipid membranes. Amyloid-β (Aβ), α-synuclein and amylin proteins have
been described to form “ion-channel-like structures” on the cell membrane, which permeates the membrane and allows calcium flux. Aβ and α-synuclein have also been shown to remodel lipid membranes through a “carpeting-effect mechanism”, where the amyloid proteins blanket the lipid membrane causing membrane thinning or generating membrane curvature. Membrane disruption can also be caused by a “detergent-like mechanism” as observed with Aβ, where the peptide removes lipids from the membrane by forming micelle-like structures with the lipids. Tau protein was also shown to extract lipids through a similar mechanism leading to the formation of stable protein/lipid complexes. Molecular details of lipid membrane destabilization caused by tau protein, however, remain poorly understood.

Tau interaction with the cell membrane is also suspected to nucleate fibrillation. Previous studies have shown that upon interaction with anionic lipid membrane, tau, Aβ, and α-synuclein are more prone to fibril formation. Possible explanations for accelerated fibril nucleation include membrane-induced changes to protein structure, partial folding of the intrinsically disordered proteins via membrane association or penetration, and increased protein concentration at the membrane. In tau protein, three segments located in the MTB domain (253-261, 315-323, and 346-355) have been found to bind to lipid membranes and adopt a helical structure which promotes protein aggregation by enhancing protein-protein interactions.

In this study, we investigated the molecular level structural changes associated with the insertion of two PHF6 isoforms into lipid membranes and explored the effects on membrane stability and peptide fibrillation. After characterizing fibrillation of both peptides, their neuronal cell toxicity was evaluated. Angstrom level structural changes associated with PHF6
insertion into a lipid monolayer membrane at the air/water interface were investigated by in situ synchrotron X-ray reflectivity (XR) and grazing incidence X-ray diffraction (GIXD). The membrane-mediated conformational changes and fibrillation of PHF6 were also characterized in the presence of large unilamellar vesicles (LUVs). We found that both peptides preferentially inserted into the negatively charged lipid membrane which led to different conformational changes; NH$_3^+$-PHF6 inserted into the lipid membrane while Ac-PHF6 formed a layer at the surface. Despite different macroscale events, both PHF6s disrupted membrane stability and underwent membrane-mediated fibrillation.

### 3.3 Results

PHF6 is an amphiphilic hexapeptide(32) located in the third repeat unit of the tau MTB (Figure 3-1A). This hexapeptide has been shown to drive tau fibrillation by forming the core of β-sheet rich tau aggregates(11-13). In this study, we characterized the structural changes associated with PHF6 upon interaction with lipid membranes and investigated its role in cell toxicity and peptide fibrillation. Two PHF6 isoforms were evaluated, non-acetylated PHF6 (NH3+-PHF6) and N-terminal acetylated PHF6 (Ac-PHF6) (Figure 3-1B and 1C), to investigate the influence of peptide charge on fibrillation and membrane interaction.
Figure 3-1: Structures of hTau40 protein and PHF6 peptides. (A): hTau40, made of 411 amino acids, is the longest isoform found in the brain. It is composed of a projection domain (green) and microtubule binding domain (blue). PHF6 is a hexapeptide found in the third repeat unit of the microtubule binding domain (R3) and is amphiphilic. (B) and (C): Structures of $\text{NH}_3^+$-PHF6 and Ac-PHF6 peptides. The acetyl group, the positive and negative charges are indicated in black, blue, and red, respectively.

3.3.1 N-acetylation of PHF6 promotes fibrillation

PHF6 has been previously described to form fibrillar aggregates rich in parallel $\beta$-sheets$^{101,102}$. In this study we evaluated the capability of $\text{NH}_3^+$-PHF6 (net charge at pH 6: +1) and Ac-PHF6 (net charge at pH 6: 0) to form fibrils by incubating the peptides in MilliQ water at 3 mg/mL at 70°C for 3 days.

As shown by TEM images in Figure 3-2A and C, unincubated $\text{NH}_3^+$-PHF6 and Ac-PHF6 showed very few features indicating that both peptides are soluble and likely monomeric. After 3 days of incubation at 70°C, only Ac-PHF6 formed characteristic amyloid fibrils (Figure 3-2D) while $\text{NH}_3^+$-PHF6 remained unaggregated (Figure 3-2B). These results are consistent with previous findings$^{101}$ and indicate that Ac-PHF6 is more prone to fibrillation.
than NH$_3^+$-PHF6, which could be due to electrostatic repulsions between positively charged NH$_3^+$-PHF6 peptides.

![Figure 3-2: TEM images of NH$_3^+$-PHF6 and Ac-PHF6 before and after 3 days of incubation at 70 °C at 3 mg/mL under quiescent condition. Both PHF6 peptides appear monomeric when unincubated and only Ac-PHF6 formed mature fibrils after 3 days of incubation.](image)

3.3.2 **PHF6 peptides are cytotoxic**

We next evaluated neuronal toxicity of unincubated NH$_3^+$-PHF6, unincubated Ac-PHF6, and pre-formed Ac-PHF6 fibrils on cultured neuroblastoma SH-SY5Y cells by MTS assay. Normalized viability results are reported in **Figure 3-3**. Compared to untreated cells (control), cells treated with unincubated and fibrillar PHF6 displayed lower levels of cell viability ($p \leq 0.01$), with final viabilities lower than the 70% threshold generally considered for cytotoxicity$^{11}$. No significant difference in cell viability was observed between cells treated with unincubated NH$_3^+$-PHF6 and Ac-PHF6. Additionally, the pre-formed Ac-PHF6 fibrils exhibited highest toxicity with a viability value close to 40%.
Figure 3-3: SHSY-5Y cell viability after 48 hours of treatment with 20 µM of unincubated NH₃⁺-PHF6, unincubated Ac-PHF6, or preformed Ac-PHF6 fibrils. Cell viability was normalized to the control, untreated cells. Error bars represent standard deviations of quintuplet experiments. Red line represents 70% cell viability used as a threshold for cytotoxicity. Asterisks indicate significant differences between two conditions (t-test with a $p$-value ≤ 0.01).

To complement the MTS-based cell toxicity assay, a live/dead stain assay was performed. Two stains were used, calcein-AM and ethidium homodimer-1 (EthD1). Live cells were stained green by the conversion of the cell-permeant calcein-AM to green fluorescent calcein dye (excitation at 494 nm, emission at 517 nm). Membrane compromised cells were stained red from binding of the non-cell-permeant EthD1 to DNA that induces fluorescence turn-on (excitation at 528 nm, emission at 617 nm). After incubating the neuroblastoma cells with 10 µM unincubated NH₃⁺-PHF6, unincubated Ac-PHF6, or fibrillar Ac-PHF6 for 24 hours, fluorescence images were taken (Figure 3-4A) and the number of dead cells was quantified (Figure 3-4B). Compared to the untreated control, all PHF6 samples caused significant increases in dead counts (cells/mm²) ($p ≤ 0.01$), indicating that PHF6 caused
membrane permeation which could contribute to the peptide’s toxicity. Comparable results were obtained for both unincubated NH$_3$$^+$-PHF6 and Ac-PHF6, indicating no significant difference in their membrane permeation activity. Additionally, the β-sheet rich fibrils caused higher dead cell counts (Figure 3-2) than the unincubated peptides. Taken together, our results show that both NH$_3$$^+$-PHF6 and Ac-PHF6 are cytotoxic and the fibrillar state is more toxic and membrane disruptive compared to the unaggregated state.

**Figure 3-4:** Live/dead staining assay results of SHSY-5Y cells incubated with 10 µM unincubated NH$_3$$^+$-PHF6, unincubated Ac-PHF6, or preformed Ac-PHF6 fibrils. (A): Fluorescence images of SHSY-5Y cells exposed to PHF6. Green and red cells correspond to live cells (calcein-AM staining) and dead cells (EthD1 staining), respectively. The red arrows indicate the dead cells. (B): Quantitative results of live/dead assay images, where dead cells were counted from 4 fluorescence images taken for each treatment condition. Asterisks represent significant differences between two conditions (t-test with a p-value ≤ 0.01).

3.3.3 **PHF6 inserts into and destabilizes lipid membranes**

One hypothesized mechanism of amyloid protein toxicity is the disruption of cell membrane structure and function via direct protein-membrane interactions. Indeed, the live/dead staining assay results show a correlation between PHF6 peptide toxicity and membrane permeabilization. To characterize PHF6 interactions with the cell membrane, we
used lipid monolayers formed at the air/water interface in a Langmuir trough as a model membrane system. Two lipids were used, zwitterionic DMPC and anionic DMPG. The insertion of NH$_3^+$-PHF6 or Ac-PHF6 into monolayers was measured while the monolayer surface area was held constant (Figure 3-5). As such, favorable interactions that cause tau to insert into the lipid monolayer led to increases in surface pressure.

Both PHF6 peptides readily inserted into anionic DMPG monolayers, causing initial fast increases in surface pressure after injecting the peptides into the subphase (Figure 3-5). Consistent with previously published findings with hTau40$^{29}$, the truncated Ac-PHF6 did not insert into zwitterionic DMPC monolayer (data not shown), indicating that the peptide preferentially interacts with anionic lipid membranes. The insertion of NH$_3^+$-PHF6 resulted in higher surface pressure increases than Ac-PHF6 (Figure 3-5A). As both peptides exhibited similar adsorption to a clean air/water interface (Supplementary Figure S1), the difference in membrane insertion was likely due to differences in their specific interactions with the lipid membrane rather than their intrinsic surface activities. After NH$_3^+$-PHF6 rapidly inserted into the membrane, a slow but persistent decrease in surface pressure was observed over time, indicating that some material was being removed from the surface (lipids and/or peptides). Note that this pressure loss is not due to instability of the lipid film, as lipid monolayers held at 25 mN/m did not show appreciable pressure loss over time (data not shown). Interestingly, Ac-PHF6 displayed two unique phases of surface pressure loss after the initial rapid increase: a sharp decrease in surface pressure, followed by a slower loss of about 2 mN/m per hour. This two-phase surface pressure loss might be caused by different mechanisms of membrane destabilization.
To monitor lipid monolayer morphology, fluorescence microscopy (FM) was used to image the monolayer before and after peptide insertion (Figure 3-5B and Figure 3-5C); 0.5 mol% headgroup labeled lipid dye Texas-Red DHPE (TR-DHPE) was included in the monolayer for FM imaging. A DMPG monolayer compressed to 25 mN/m has undergone phase transition where ordered liquid-condensed domains (LC) domains formed in the disordered liquid-expanded (LE) phase. As the bulky fluorescent dye was excluded from the LC phase, it appeared as dark patches while LE phase appeared bright.

The insertion of NH$_3^+$-PHF6 caused negligible changes to the monolayer morphology as FM images appeared similar before and at different time points of peptide insertion (Figure 3-5B). In contrast, insertion of Ac-PHF6 resulted in drastic changes to monolayer morphology, where at least three fluorescence intensity levels were observed in FM images (Figure 3-5C). In addition to the bright LE and dark LC phases, a third even brighter phase was formed in the LE portion of the film at earlier time points (Figure 3-5C, 15-45 min). These brighter features could be membrane invagination$^{112}$ caused by the insertion of Ac-PHF6. After 1 hour of incubation, the third bright phase disappeared and the dark LC domains became progressively reduced, which could be due to the disruption of these domains by the peptide or reduction of the monolayer surface pressure observed with time.
Figure 3-5: Isotherms (A) and fluorescence microscopy (FM) images (B and C) of NH\textsubscript{3}+\textendash PHF6 and Ac-PHF6 insertion into DMPG monolayer at the air/water interface under constant area conditions. 0.5 mol\% fluorescent dye Texas Red-DHPE was included in the DMPG monolayer. Contrast in the FM images arises from the dye being excluded from liquid-condensed (LC) domains (shown as dark patches), making the liquid-expanded (LE) fluid lipid phase bright. Interestingly, three different levels of brightness were observed in FM images of Ac-PHF6 insertion, where the LC (dark) and LE (gray) are in coexistence with a brighter phase during earlier time points (15-45min) of Ac-PHF6 insertion.

One cause for the higher level of insertion of NH\textsubscript{3}+\textendash PHF6 compared to Ac-PHF6 could be the different overall peptide charges due to acetylation. At pH 6.0 (approximate water pH), NH\textsubscript{3}+\textendash PHF6 had a net positive charge that promoted its insertion into the negatively charged lipid membrane. In contrast, Ac-PHF6 had a neutral charge and therefore had less attraction to the anionic DMPG lipid headgroups. Overall, both peptides favorably interacted with the anionic membrane and extracted material (lipids, peptides, or both) from the membrane. Additionally, our results show that although Ac-PHF6 inserted less into the membrane than NH\textsubscript{3}+\textendash PHF6, it caused faster and more disruptions to the membrane stability and morphology.

Additional, our results show that although Ac-PHF6 inserted less into the membrane than NH\textsubscript{3}+\textendash PHF6, it caused faster and more disruptions to the membrane stability and morphology. The peptides favorably interact with anionic lipid monolayers and some differences are seen on their effects on membrane morphology. To gain further insights into the molecular level
structural changes of the peptides and the lipid monolayer accompanying PHF6 insertion, we used in situ synchrotron X-ray scattering techniques to determine the angstrom-level structure and organization of lipids and tau peptides at the air/water interface.

X-ray reflectivity (XR) was employed to determine the electron density distribution of lipid-peptide films at the air/water interface in the direction perpendicular to the film surface (z-direction) with sub-angstrom resolution. Using this method one can distinguish between regions of materials with different electron densities such as, in our case, phospholipid tails, lipid headgroups, peptides, or mixtures of these components. This information can be used to reconstruct a model depicting the structure of the phospholipid/peptide film in the z-direction (averaged over the coherent volume of the X-ray beam) and determine the location of peptide in the membrane as previously described. A DMPG monolayer at 25 mN/m was modeled as two “slabs” of different electron densities (Figure 3-6A1 and Figure 3-6A2 and Supplementary Table S3) – a 15.9 ± 0.2 Å thick slab of phospholipid tails at a normalized electron density ($\rho/\rho_{water}$) value of 0.98 ± 0.03 and a 9.1 ± 0.3 Å thick slab of more electron dense phospholipid heads with a $\rho/\rho_{water}$ value of 1.58 ± 0.02. The insertion of NH$_3^+$-PHF6 into the lipid monolayer induced subtle structural changes to the DMPG monolayer (Figure 3-6).

At 2.5 hours after injection, $\rho/\rho_{water}$ of the DMPG lipid head groups decreased from 1.58 ± 0.02 (Figure 3-6A2) to 1.49 ± 0.03 (DMPG + NH$_3^+$-PHF6) (Figure 3-6B2). This change could be caused by the insertion of NH$_3^+$-PHF6 into the lipid headgroups, thus decreasing the $\rho/\rho_{water}$ value of this layer. The $\rho/\rho_{water}$ values of proteins, including Aβ40 and hTau40, adsorbed to an air/water interface have been previously reported to be around 1.26$^{29,31}$. Thus, the intermediate $\rho/\rho_{water}$ value of 1.49 ± 0.03 (Figure 3-6B2) is likely a mix of peptides and lipid headgroups.

Concurrently, $\rho/\rho_{water}$ of the lipid tails increased slightly from 0.98 ± 0.03 to 1.02 ± 0.004,
suggesting some peptide insertion into the tails. A third slab was modeled beneath the lipid head groups of electron density that was subtly distinguishable from the water subphase \((\rho/\rho_{\text{water}} \text{ value of } 1.041\pm 0.005)\). This indicated a sparsely populated layer of NH\textsubscript{3}\textsuperscript{+}-PHF6 adsorbed to the lipid head groups. A similar observation was made at 4.5 and 6.5 hours – a small change in electron density indicated NH\textsubscript{3}\textsuperscript{+}-PHF6 insertion into the membrane and a sparse layer of adsorbed peptide.

**Figure 3-6:** X-ray reflectivity (XR) results of NH\textsubscript{3}\textsuperscript{+}-PHF6 interacting with a DMPG monolayer. (A1-D1): Normalized Fresnel reflectivity \((R/R_F)\) profiles of a DMPG monolayer before (A1) and 2.5 (B1), 4.5 (C1), and 6.5 (D1) hours after injecting NH\textsubscript{3}\textsuperscript{+}-PHF6 into the subphase. Experimental data are plotted as points with errors, and the fitted model is overlaid as solid lines. (A2-D2): Electron density profiles normalized to that of water \((\rho/\rho_{\text{water}})\) for a DMPG monolayer before (A2) and 2.5 (B2), 4.5 (C2), and 6.5 (D2) hours after injecting NH\textsubscript{3}\textsuperscript{+}-PHF6 into the subphase. The depth, or distance in the \(z\)-direction, is plotted with zero marking the air/lipid tails interface; negative values are in the air and positive values extend into the water subphase. Solid lines represent slab model fits and the dashed lines represent smoothed slab model fits where the slabs were fit with roughness at interfaces. Lipids and NH\textsubscript{3}\textsuperscript{+}-PHF6 (green) schematics are included to help visualize the molecular layers that gave rise to the fitted \(\rho/\rho_{\text{water}}\) profiles. Larger-scale schematics of the membrane structure without (A2) or with NH\textsubscript{3}\textsuperscript{+}-PHF6 inserted into the membrane (B2-D2) are shown in the upper left hand corner of the electron density profiles.
In contrast, insertion of Ac-PHF6 induced drastic changes to the lipid monolayer structure (Figure 3-7 and Supplemental Table S4). After 2.5 hours of Ac-PHF6 incubation with the monolayer, the membrane had clearly lost many features of its original structure. Three slabs were needed to fit the XR reflectivity data (Figure 3-7B2). The two slabs representing the lipid monolayer had decreased electron densities with increased roughness values indicating less distinct layers. Combined with the drastic decreases in surface pressure observed after the addition of Ac-PHF6 (Figure 3-5), the decrease in electron density was likely due to loss of lipids at the surface. A third layer, extending 24.1 ± 0.07 Å into the water subphase at a $\rho/\rho_{\text{water}}$ value of 1.21 ± 0.0014 is likely a peptide layer forming underneath the membrane (Figure 3-7B2). This highly perturbed monolayer structure could be the result of large-scale membrane deformations, such as invagination, which were also observed by FM imaging (Figure 3-5C). At 4.5 hours, the membrane-peptide film resembled a typical lipid monolayer with a less prominent peptide layer underneath the lipid headgroups (16.3 ± 0.3 Å thick with a $\rho/\rho_{\text{water}}$ value of 1.06 ± 0.0018). The electron density of the lipid membrane layers decreased by about 10% compared to that at 25 mN/m, reflecting the loss of material at the surface. Finally, at 6.5 hours the lipid/peptide film structure was similar to that at 2.5 hours – a highly perturbed lipid monolayer covered with a thick layer of bound peptides.
3.3.5 PHF6 forms β-sheet rich structures and disrupts lipid packing

While analysis of XR data provides electron density distributions averaged over both in-plane ordered and disordered parts of the monolayer, grazing incidence X-ray diffraction (GIXD) detects only ordered diffracting species present at the air/water interface. As the expected ordered structures included condensed phospholipid tails and repeating peptide structures such as β-sheet-rich fibrillar structures, GIXD was used to monitor assembly of PHF6 peptides and its impacts on lipid packing. Fitting of integrated diffraction peaks gave rise to the lattice spacing (d-spacing), coherence length (Lc), and relative amounts of diffracting material in the plane of the lipid/peptide film. Lc is calculated from the full width at half
maximum peak intensity (FWHM) and $L_c$ value represents the average size of diffracting crystallites. GIXD diffraction images and a summary of the peak fitting parameters are reported in Supplementary Material Figure S2, Tables S1 and S2.

DMPG monolayer at 25 mN/m showed a single diffraction peak indicating hexagonally packed lipid tails in the LC phase with a distance between acyl tails of $4.87 \pm 0.02$ Å (Table 3-1) and a $L_c$ value of $513 \pm 15$ Å (Table 3-1 and Figure 3-8A). One hour after NH$_3^+$-PHF6 injection, the ordered lipid tails adopted a distorted hexagonal packing as two diffraction peaks were observed. At 3.5 hours, lipid tails again became hexagonally packed. However, the total amount of diffracting lipids decreased by ~60% based on peak intensity decrease. The in-plane lipid packing remained largely the same for the rest of the experiment and no other ordered structures were observed.

In contrast, the interaction of Ac-PHF6 with DMPG was more dynamic (Figure 3-8B, and Table 3-1 and Table 3-2). At one hour after Ac-PHF6 injection, the lipid diffraction peak shifted slightly to indicate larger $d$-spacing between lipid tails. The phospholipid diffraction peak also broadened significantly, corresponding to a more than 5-fold reduction of $L_c$ from ca. 500 to $73 \pm 11$ Å. Concurrently, a second, non-lipid diffraction peak appeared at a $q_{xy}$ value of $1.33$ Å$^{-1}$ that corresponds to a $d$-spacing of $4.74 \pm 0.02$ Å (Table 3-2). As this $d$-spacing exactly matches the distance between β-strands of amyloid fibers measured by X-ray diffraction$^{113}$, the appearance of this peak indicates the formation of semi-crystalline β-strand lattice structures of Ac-PHF6 stabilized by hydrogen bonds. The $L_c$ calculated from the peptide diffraction peak was $125 \pm 19$ Å, indicating that ca. 26 peptides are in positional registry in the crystallites. At 3.5 hours, two lipid diffraction peaks were observed, indicating distorted hexagonal packing with tail-tail intermolecular distances of $4.841 \pm 0.016$ and $4.966 \pm 0.018$
Å with corresponding $L_c$ values of $340 \pm 50$ and $95 \pm 14$ Å, respectively. Interestingly, at 3.5 hours, the peptide diffraction peak diminished significantly before returning to a high intensity peak at 5 hours. At 5 hours the lipid diffraction indicated a return to hexagonal packing with small ordered lipid domains ($L_c$ of $76 \pm 11$ Å), and at 7 hours both the lipid and peptide diffraction peaks were diminished significantly.

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We additionally extracted Bragg rod data by integrating X-ray diffraction images over $q_{xy}$ to determine the length of the coherently scattering molecular entity along the $z$-dimension ($L_m$). Analysis of the Ac-PHF6 diffraction peak at 1.33 Å$^{-1}$ at 5 hours after peptide injection yielded a $L_m$ value of $20.2 \pm 0.6$ Å (Supplementary Figure S4). Thus, of the $24.12 \pm 0.07$ Å thick peptide layer accumulated underneath the lipid headgroups as detected by XR, 20.2 Å of it was participating in diffraction.

Taken together, GIXD resolved three distinct stages of the Ac-PHF6 membrane film after peptide addition. The first is low intensity diffraction from hexagonal lipid packing with strong peptide diffraction observed 1.0 hour post injection. At this stage, Ac-PHF6 is bound to the DMPG monolayer, and the binding disrupted lipid packing and induced the peptide to assemble in β-sheet rich crystalline aggregates. This stage is followed by a second distinct stage of high intensity distorted hexagonal lipid packing with little peptide diffraction at 3.5 hours. The interpretation of this drastic change in the peptide-lipid film is that as the β-sheet rich Ac-PHF6 aggregates grew, they detached from the lipid monolayer and resulted in a diminished peptide diffraction peak at the air/water interface. This loss of peptides from the interface is corroborated by observed loss of surface pressure and changes in membrane morphology (Figure 3-5). At this stage, another cycle of Ac-PHF6 insertion and assembly occurred (5.0-6.5 hours), leading to detachment and loss of material at the interface at 7.0 hours.
post injection where significant surface pressure loss and only a weak lipid diffraction peak were observed.

**Figure 3-8**: Grazing incidence X-ray diffraction (GIXD) results of Ac-PHF6 and NH$_3^+$-PHF6 interacting with DMPG lipid monolayers. Background-subtracted integrated diffraction values (points) are overlaid with the Voigt model fit to the data (solid lines). Error bars are shown in gray. (A): Bragg peaks from time-dependent NH$_3^+$-PHF6 interactions with a DMPG lipid monolayer. Only lipid diffraction peaks around 1.48 Å$^{-1}$ from condensed lipid tail packing were observed. (B): Bragg peaks obtained from Ac-PHF6 interactions with a DMPG monolayer over time. The new peaks around 1.32 Å$^{-1}$ are attributed to peptide diffraction from in-registry β-sheets. (C): GIXD peaks from Ac-PHF6 interactions with DMPG 5 hours after peptide injection. No other samples showed peaks in this region.
Table 3-1: GIXD fitting parameters obtained from DMPG lipid diffraction peaks after injection of NH$_3^+$-PHF6 or Ac-PHF6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface Pressure</th>
<th>Tail-Tail Intermolecular</th>
<th>Integrate d Peak</th>
<th>Coherence Length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NH$_3^+$-PHF6 Lipid Diffraction Peaks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPG</td>
<td>25.2</td>
<td>4.87 ± 0.02</td>
<td>668 ± 12</td>
<td>510 ± 80</td>
</tr>
<tr>
<td>DMPG + NH$_3^+$-PHF6 – 1.0 h</td>
<td>30.0</td>
<td>4.96 ± 0.02</td>
<td>820 ± 50</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>DMPG + NH$_3^+$-PHF6 – 3.5 h</td>
<td>31.7</td>
<td>4.85 ± 0.02</td>
<td>277 ± 9</td>
<td>580 ± 90</td>
</tr>
<tr>
<td>DMPG + NH$_3^+$-PHF6 – 5.0 h</td>
<td>30.8</td>
<td>4.84 ± 0.02</td>
<td>246 ± 8</td>
<td>600 ± 90</td>
</tr>
<tr>
<td>DMPG + NH$_3^+$-PHF6 – 7.0 h</td>
<td>28.6</td>
<td>4.84 ± 0.02</td>
<td>310 ± 8</td>
<td>580 ± 90</td>
</tr>
<tr>
<td><strong>Ac-PHF6 Lipid Diffraction Peaks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPG</td>
<td>28.9</td>
<td>4.870 ± 0.015</td>
<td>890 ± 40</td>
<td>480 ± 80</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 1.0 h</td>
<td>37.3</td>
<td>4.912 ± 0.017</td>
<td>1200 ± 100</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 3.5 h</td>
<td>33.3</td>
<td>4.966 ± 0.018</td>
<td>1210 ± 60</td>
<td>95 ± 14</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 5.0 h</td>
<td>30.1</td>
<td>4.841 ± 0.016</td>
<td>370 ± 30</td>
<td>340 ± 50</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 7.0 h</td>
<td>26.3</td>
<td>4.897 ± 0.017</td>
<td>860 ± 60</td>
<td>76 ± 11</td>
</tr>
</tbody>
</table>

*A conservative 15% error is assumed for $L_c$ values

From the GIXD data, we additionally deduced tertiary structural information about the Ac-PHF6 β-sheet rich aggregates (or protofibrils). In GIXD the X-rays travel in the plane of the lipid monolayer and they only diffract from ordered species with their unit cell perpendicular to that plane. Thus, the β-strand unit cell must be oriented perpendicular to the lipid monolayers, which suggests that the fibril growth axis was parallel to the lipid membrane (**Figure 3-9F**). At 5.0 hours, two additional diffraction peaks were observed at $q_{xy}$ values below 0.5 Å$^{-1}$ (**Figure 3-8C**), with $d$-spacings of 9.0 ± 0.06 and 11.5 ± 0.13 Å. Although the exact
structures that gave rise to these peaks are unknown, we posit that at least one of the peaks arose from inter-β-sheet packing of the protofibril as amyloid fibrils have been reported to have an approximate spacing of 10 Å between β-sheets\(^{13}\). Thus, these diffraction peaks may have arisen from two types of inter-sheet packing arrangements. Alternatively, the diffraction could be from ordered phospholipid headgroups templated by electrostatic interactions with the protofibril. Although lipid headgroups diffraction is typically not observed by liquid surface X-ray scattering experiments, this study may be a unique case driven by the high degree of ordering in the bound protein protofibrils and their strong interaction with the negatively charged head groups via positively charged lysine moieties. These observations, along with the XR data, corroborate dynamic Ac-PHF6-membrane interactions and structural reorganization as described previously.

**Table 3-2:** GIXD fit parameters of Ac-PHF6 diffraction peaks after injecting the peptide underneath a DMPG monolayer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>d-Spacing (Å)</th>
<th>Integrated Peak Area</th>
<th>Coherence Length (L_c) (Å)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPG + Ac-PHF6 – 1.0</td>
<td>4.74 ± 0.02</td>
<td>770 ± 40</td>
<td>125 ± 19</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 3.5</td>
<td>4.73 ± 0.03</td>
<td>150 ± 30</td>
<td>86 ± 13</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 5.0</td>
<td>4.73 ± 0.03</td>
<td>770 ± 40</td>
<td>210 ± 30</td>
</tr>
<tr>
<td></td>
<td>11.5 ± 0.3</td>
<td>43 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.01 ± 0.14</td>
<td>130 ± 20</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>DMPG + Ac-PHF6 – 7.0</td>
<td>4.74 ± 0.02</td>
<td>280 ± 30</td>
<td>125 ± 19</td>
</tr>
</tbody>
</table>

*A conservative 15% error is assumed for \(L_c\) values.

With the findings from GIXD and XR, we constructed a detailed structural model for PHF6 peptide interaction with the anionic lipid membrane (Figure 3-9). Favorable
electrostatic interactions cause $\text{NH}_3^+$-PHF6 to fully insert into the phospholipid monolayer, as evidenced by modulated electron density of the headgroups and tails. A sparsely populated peptide layer was observed outside of the DMPG monolayer. As a result of peptide interaction, packing of lipid tails became distorted (Figure 3-9A and Figure 3-9C). Ac-PHF6 first interacted with DMPG to form a 16 Å thick layer of peptides underneath the lipid headgroups and induced distorted hexagonal packing of the lipid tails (Figure 3-9D). This layer was possibly formed by burying the C-terminal cationic lysine in the anionic headgroups, leaving 16 Å of peptide to protrude below the lipid monolayer (Figure 3-9B and Figure 3-9D). Ac-PHF6 quickly formed β-sheet rich protofibrillar structures after inserting into the DMPG membrane as evidenced by a β-strands $d$-spacing of 4.7 Å (Figure 3-9F). These protofibrils are oriented with the growth axis parallel to the membrane, and likely had one terminal lysine inserted into the lipid headgroups. In addition to the β-strands $d$-spacing of 4.7 Å, two additional $d$-spacings of 9.0 and 11.5 Å were also observed between the β-sheets (Figure 3-9F). The 24 Å thickness of the peptide layer under the monolayer was consistent with the PHF6 fibril structure with one terminal lysine excluded from the measurement (Figure 3-9 E/F and Supplementary Figure S3). This model was further corroborated by Bragg rod analysis of the 5.0 hours Ac-PHF6 1.328 Å$^{-1}$ diffraction peak (Supplementary Figure S4). The result was that 20.2 ± 0.6 Å of the peptide layer were contributing to the diffraction peak out of the 24 Å protein layer measured by XR studies.
Figure 3-9: Model for PHF6 interactions with DMPG monolayer based on XR and GIXD data. (A): $\text{NH}_3^+$-PHF6 structure extracted from a previously published structure of a paired helical filament\textsuperscript{114}. Positive charges on the peptide are circled in blue. The length of the peptide (red) excluding the C-terminal lysine is estimated. (B): Ac-PHF6 structure with the positive charge circled in blue (blue). (C): Model for non-fibrillar (unincubated) $\text{NH}_3^+$-PHF6 interacting with a DMPG monolayer. Electrostatic interactions drive the insertion of the positively charged peptide into the anionic DMPG monolayer. Lipid tails are in a distorted hexagonal packing structure (D): Model for unincubated Ac-PHF6 interactions with DMPG. Peptide inserts with the cationic lysine (blue) into the anionic DMPG headgroup. A 16 Å layer of peptide is observed below the lipid monolayer, and lipids are in a distorted hexagonal structure. (E): Model of an Ac-PHF6 fibril derived from a previously published fibril structure (Supplemental Figure S3)\textsuperscript{114}. 2 peptides are shown here. Measured distance (red) excludes one terminal lysine, assuming that the lysine is embedded in the DMPG head groups. Positive charges are circled in blue. (F): Model for inserted and assembled Ac-PHF6 protofibril interacting with DMPG monolayer. Lipid packing is disrupted due to peptide interactions, and one terminal lysine is assumed to insert into the anionic phospholipid head group. The fibril forms a 24 Å layer of peptide beneath the lipids. The fibril growth axis is parallel to the plane of the lipids, and there is a lattice spacing of 4.7 Å between β-strands, and 9.0 or 11.5 Å spacing between β-sheets.

3.3.6 PHF6 interaction with LUVs promotes fibrillation

$\text{NH}_3^+$-PHF6 and Ac-PHF6 displayed selective favorable interactions with anionic DMPG membrane and Ac-PHF6 was also shown to assemble into β-sheet rich protofibrils
upon interaction. In order to investigate the effect of this interaction on peptide fibrillation, both hexapeptides were incubated in water with 100 nm large unilamellar vesicles (LUVs) composed of 70 mol% POPC and 30 mol% POPG. Peptide secondary structures, vesicle size distribution, and formation of fibrils were evaluated using circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), and TEM imaging, respectively. We did not use the popular fluorescence probe Thioflavin T to track fibril formation because of its poor binding to PHF6 fibrils.

Freshly solubilized NH$_3^+$-PHF6 and Ac-PHF6 were rich in random coils as shown by the negative peak at around 195 nm in CD spectra (Figure 3-10). After 1-hour incubation with vesicles (168 µg/mL peptide with 688 µM lipids) at room temperature, drastic changes in secondary structure were observed for both peptides; positive and negative peaks at around 200 nm and 218 nm, respectively, appeared, indicating the formation of β-sheet rich structures. A stronger CD signal was observed in the case of Ac-PHF6 showing a higher β-sheet content compared to NH$_3^+$-PHF6. The formation of β-sheets by Ac-PHF6 corroborates our finding from GIXD measurements (Figure 3-8). However, GIXD did not detect β-sheet assembly of NH$_3^+$-PHF6 at the pure DMPG membrane surface. CD is a bulk solution technique, thus, the measured β-sheet content in NH$_3^+$-PHF6 incubated with LUVs could be from the peptide adopting a β-sheet conformation in solution, and not at the surface of the membrane. As NH$_3^+$-PHF6 was shown to destabilize the lipid monolayer (Figure 3-5), it is likely that the peptide could adopt a β-sheet conformation when complexed with dissolved lipids. Alternatively, the membrane curvature found in LUVs may induce β-sheet structures more than what is observed in the flat lipid monolayer system.$^{36,38}$
Figure 3-10: CD spectra of 168 µg/mL NH₃⁺-PHF6 and Ac-PHF6 peptides alone (168 µg/mL) (solid line) and in the presence LUVs (688 µM lipids, 70 mol% POPC and 30 mol% POPG) after 1 hour incubation at room temperature (dashed line). LUVs caused the largely random coil peptides to adopt β-sheet rich structures.

LUV size distribution was monitored by DLS during incubation with both peptides (50 µg/mL PHF6 in the presence of 20 µM lipids) (Figure 3-11). LUVs were stable for over 7 days at room temperature where hydrodynamic radius ($R_h$) remained unchanged at ~50 nm (Figure S5). The addition of NH₃⁺-PHF6 to the LUVs did not induce any changes to the vesicle size distribution during the first 24 hours, but the size distribution became broader and $R_h$ increased after 6 days (Figure 3-11A). By day 8, the vesicles likely became unstable and agglomerated. In contrast, after mixing with Ac-PHF6, an immediate broadening of LUV size distribution and increase of $R_h$ were observed (Figure 3-11B). By day 6, size distribution shifted towards larger sizes and became very broad. These changes are likely caused by immediate peptide binding and vesicle agglomeration, followed by peptide fibrillation at longer incubation times.
Figure 3-11: Size distribution of LUVs (20 µM lipids, 70 mol% POPC and 30 mol% POPG) incubated at room temperature in the presence of 50 µg/mL NH$_3^+$-PHF6 (A) or Ac-PHF6 (B) over time as detected by DLS. LUVs size distribution was unchanged after 1 day of incubation with NH$_3^+$-PHF6 and became larger after 6 days of incubation. In contrast, Ac-PHF6 induced rapid increase of $R_h$ and broader LUV size distribution immediately upon addition.

To further understand the increase of the LUV size induced by NH$_3^+$-PHF6 and Ac-PHF6 and the changes in secondary structures, the samples were imaged by TEM (Figure 3-12). The sample containing NH$_3^+$-PHF6 incubated with LUVs for 1 hour showed round, 100-200 nm-sized features (Figure 3-12A). These are likely peptide-coated LUVs as bare lipid vesicles are completely disrupted during the drying step in sample preparation for TEM.$^{31}$ After 6 days of incubation, long fibrils were visible, which supports DLS measurements that showed the presence of larger structures in the sample. When incubated alone, NH$_3^+$-PHF6 did not form fibrils in the same incubation period, indicating that the interaction with the lipid membrane promoted NH$_3^+$-PHF6 fibrillation, possibly by reducing the repulsive electrostatic interactions between positively charge ammonium groups of NH$_3^+$-PHF6. Also, by binding to the lipid membrane, the increased local concentration of NH$_3^+$-PHF6 is also expected to promote peptide fibrillation.
In the presence of LUVs, Ac-PHF6 produced long fibrils after only a few hours of incubation (Figure 3-12B), which is consistent with the high β-sheet content measured by CD spectroscopy (Figure 3-10) and by GIXD (Figure 3-8). Fibrils produced in the presence of LUVs formed large clusters connected to small dark agglomerates, which appear to be Ac-PHF6-coated-LUVs. In contrast, when the same peptide was incubated alone, significantly slower fibrillation was observed; short protofibrils were observed after 1 hour and long fibrils were only observed after 6 days of incubation. Taken together, our results show that anionic vesicles accelerated fibrillation of both NH₃⁺-PHF6 and Ac-PHF6.

Figure 3-12: TEM images of NH₃⁺-PHF6 (A) and Ac-PHF6 (B) incubated alone (100 µg/mL) and in the presence of LUVs (40 µM) over 6 days at room temperature under quiescent condition.
3.4 Discussion

The interaction of the tau protein with the lipid membrane is believed to play a key role in peptide toxicity and fibrillation. In this study, we investigated the lipid membrane interaction of PHF6, a hexapeptide located in the third repeat unit of the MTB domain forming the core of tau PHFs and revealed its effect on membrane structure and peptide fibrillation. We used two PHF6 peptides, one fibrillation prone (Ac-PHF6) and another not fibrillation prone (NH$_3^+$-PHF6), to study the effect of these peptides on cell toxicity, membrane interaction, and membrane-mediated fibrillation.

We found that both unincubated PHF6s are neurotoxic and pre-formed Ac-PHF6 fibrils exert higher toxicity (Figure 3-3). Membrane disruption via direct peptide interactions has been proposed as a mode of toxicity for amyloidogenic proteins, and is the central hypothesis tested in this work. Membrane destabilization caused by PHF6 is supported by Langmuir trough isotherm experiments where both Ac-PHF6 and NH$_3^+$-PHF6 favorably interact with anionic, but not zwitterionic, lipid monolayers at the air/water interface (Figure 3-5). This interaction leads to lipid extraction from the interface into the aqueous subphase as observed by a decrease in the membrane surface pressure after the insertion of PHF6. This model agrees with a previously published study showing that full length tau inserts into negatively charged lipid membranes and extracts lipids from the membrane. Furthermore, the interaction of PHF6 with lipid membranes also enhances fibrillation kinetics of both peptides. Strikingly, in the presence of anionic LUVs, Ac-PHF6 formed fibrils within one hour of incubation, compared to 6 days required for complete fibrillation in the absence of vesicles. Likewise, the non-aggregating NH$_3^+$-PHF6 was able to form fibrils after 6 days of incubation with LUVs. Based on these results, we propose a 3-step model for PHF6 membrane-mediated toxicity: (1)
peptide insertion into the lipid membrane, (2) membrane destabilization through lipid extraction and (3) membrane-mediated fibrillation.

Despite a similar membrane-mediated toxicity mechanism, NH$_3^+$-PHF6 and Ac-PHF6 undergo different molecular-level changes when interacting with the lipid membrane, which are summarized in Figure 3-13. XR data (Figure 3-6) indicate that the cationic NH$_3^+$-PHF6 fully and rapidly inserts into the negatively charged DMPG monolayer and does not immediately form β-sheets at the membrane surface but forms fibrils within six days (Figure 3-11 and Figure 3-12). Binding of the positively charged peptides to the anionic membrane reduces the electrostatic repulsion between the peptides and increases its local concentration, both of which promote the peptide’s assembly into fibrils. In contrast, Ac-PHF6 associated with the membrane, likely via lysine-mediated electrostatic interactions, but does not fully insert into the lipid headgroups (Figure 3-9). The peptide quickly destabilizes the membrane (Figure 3-5) and assembles into β-sheet enriched protofibrils on the surface of the lipid membrane. These aggregates promote extensive membrane destabilization through both membrane invagination and fast peptide fibrillation.
Figure 3-13: Model for NH$_3^+$-PHF6 (A) and Ac-PHF6 (B) lipid membrane disruption and membrane-induced peptide fibrillation. Non-aggregation prone NH$_3^+$-PHF6 quickly inserts into lipid membrane and slightly destabilizes the membrane while promoting a slow fibrillation. In contrast, the aggregation prone Ac-PHF6 rapidly inserts into lipid membrane and quickly assemble into β-sheet rich protofibrils. Membrane destabilization and invagination occur concurrently.

It is important to note the difference in kinetics observed between experiments performed with a lipid monolayer made of pure DMPG lipids and LUVs made of 7:3 POPC:POPG lipids. In the LUV system, Ac-PHF6 reached complete fibrillation and disruption of the vesicles within one hour, but in the lipid monolayer system large changes were observed up to 7 hours. The differences in lipid compositions, membrane fluidity, membrane curvature, and peptide concentration are likely to influence both peptide interaction with lipid membranes and membrane destabilization kinetics, as observed in the case of α-synuclein$^{18}$. However, both of our experimental systems show the same trends for membrane interaction and fibrillation, while acknowledging a difference in peptide interaction kinetics.

Overall, the aggregation prone Ac-PHF6 exhibits fast membrane-mediated fibrillation and rapid membrane disruption, while the non-aggregation prone NH$_3^+$-PHF6 displays both slower membrane disruption and membrane-mediated fibrillation. Additionally, the pre-formed Ac-PHF6 fibrils displayed higher neurotoxicity than unincubated Ac-PHF6 and NH$_3^+$-PHF6 (Figure 3-3), indicating that the peptide’s assembly state and secondary structures are
involved in lipid membrane destabilization and toxicity. Also, it is important to note that the lipid models used in this study were mainly composed of negatively charged lipids that do not capture the full complexity of the cell membrane. This may account for some discrepancy between the *in vitro* experiments and the *in vivo* cell toxicity assays. The cell membrane is asymmetric with zwitterionic extracellular leaflet and anionic cytosolic leaflet. While the tau protein originates inside the cell, future investigations must also consider the extracellular lipid environment as it likely plays an important role in transmission of tau toxicity throughout the brain.

In this study, we have gained molecular-level insights about PHF6-membrane interactions and effects of these interactions on tau aggregation and membrane structure. This knowledge enhances our understand of membrane-mediated tau neurotoxicity and fibrillation. Our results, together with a previously published study, support a lipid extraction mechanism of tau induced membrane destabilization, which results in the formation of stable peptide-lipid complexes. These complexes are also likely involved in the fast peptide fibrillation observed in the presence of vesicles. The production of such complexes could be linked to transcellular propagation as cell internalization of tau aggregates is believed to induce fibrillation of intracellular tau. Further study of tau-lipid complexes can shed light on the role of tau in neurodegenerative diseases. With this study we also introduced two PHF6 models, one fibrillation prone (Ac-PHF6) and one non-aggregation prone (NH$_3^+$-PHF6). These models are useful tools to study the two toxicity events associated with amyloid proteins: membrane disruption and protein fibrillation. These PHF6 models could be used to better understand the mechanism of action of therapeutic molecules and could help elucidate the toxicity mechanisms associated with amyloid aggregates.
3.5 Experimental Procedures

3.5.1 Materials

SH-SY5Y neuroblastoma cells, Dulbecco’s Modified Eagle’s Medium (DMEM) F12 media, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin (PS) at 10,000 U/mL was purchased from Thermo Fisher Scientific (Waltham, MA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay, a tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based metabolic assay, was purchased form Promega (Madison, WI). Ethidium homodimer-1 (EthD-1) and calcein-AM were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA). Lipids [1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG)] were obtained from Avanti Polar Lipids (Alabaster, AL). Texas Red 1,2-dihexadecanoyl-sn-glycero-3-Phosphoethanoloamine triethylammonium salt (TR-DHPE) was purchased from Molecular Probes Inc. Chloroform and methanol were purchased from Millipore Sigma. A MilliQ purification system (Millipore Synergy UV) was used to purify all water used. Copper grids, 400 mesh, covered by a Formvar/Carbon film (5-10 nm) were purchased from Ted Pella (Redding, CA) and 2% aqueous uranyl acetate was purchased from Electron Microscopy Sciences (Hatfield, PA).

3.5.2 Peptide synthesis, purification and incubation

N-acetyl VQIVYK (Ac-PHF6) and non-acetylated VQIVYK (NH₃⁺-PHF6) were synthesized by a solid-phase method as previously described[101]. Peptides were purified by reverse phase HPLC (RP-HPLC) using an acetonitrile-water gradient containing 0.1% TFA. Purified PHF6 were lyophilized and stored at -70 °C until use. Peptide molecular weights were confirmed by
electrospray ionization mass spectrometry and peptide purity was determined by RP-HPLC.

Stock solutions were prepared by solubilizing the peptides in MilliQ water at 4 mg/mL and filtering with a 0.02 µm filter. PHF6 concentration was determined by absorbance measurement using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) (\( \varepsilon_{280\text{nm}} = 1195.9 \text{ cm}^{-1}\text{M}^{-1}\text{cm}^{-1} \)). \( \text{MW}_{\text{Ac-PHF6}} = 790 \text{ g/mol}, \text{MW}_{\text{NH}_3^+-\text{PHF6}} = 748 \text{ g/mol}. \) PHF6 peptides were incubated at 70 °C for 3 days at 3 mg/mL or at room temperature for up to 6 days at 100 µg/mL.

3.5.3 MTS-based cell viability assay

SH-SY5Y neuroblastoma cells at passage 20 were cultivated at 37 °C and 5 % CO\(_2\) in DMEM F12 media containing 10 % FBS and 1% PS. 96-well plates were seeded with 20,000 cells/well. Cells were incubated for 16 h to allow cell attachment prior to serum deprivation over 24 h ensuring cell synchronization to a quiescent state. Cells were then treated with 20 µM PHF6 for 48 h. After treatment, cell viability was determined by the MTS reduction assay. The assay was performed by adding 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay to each well. The conversion of MTS into formazan by metabolically active cells was monitored through absorbance at 490 nm after 3 h of incubation at 37 °C and 5 % CO\(_2\). The optical density (OD) of formazan was measured using a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). The % cell viability was calculated using Equation 1.

\[
\text{% viability} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{background}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{background}}} \times 100
\]  

(1)

The control and background samples consisted of untreated cells and unseeded wells, respectively. Each condition was tested in quadruplicates and data were statistically analyzed using JMP Pro 13 software (SAS Institute, Inc., Cary, NJ). The effect of the PHF6 on cell viability was analyzed using t-test with a p-value ≤ 0.01 and 70% cell viability was used as a threshold to define cytotoxicity.
3.5.4 *Live/dead staining assay*

SH-SY5Y cells were grown in a 6-well plate at 150,000 cells/well. After 24 h incubation at 37 °C and 5 % CO2, cells were treated with 10 µM PHF6 peptide diluted in DMEM F12 media containing 10% FBS and 1 % PS. After another 24 h, cells were incubated with 1 µM EthD-1 and 0.2 µM calcein-AM in DMEM F12 media over 30 min at 37 °C and 5 % CO2. Cells were rinsed with media and imaged using a Nikon Eclipse TS100 fluorescence microscope (Nikon Instruments, Inc., Melville, NY). Two filters blocks were used. A TRITC (G2E/C) filter cube was used to detect EthD-1 (red fluorescence) and a FITC (B2E/C) filter cube was used to detect calcein (green fluorescence). Images were then merged using Image J (National Institutes of Health, Bethesda, MD, USA). Finally, dead cell density (number of dead cells/mm²) was determined and the results were statistically analyzed using JMP Pro 13 software (SAS Institute, Inc., Cary, NJ) based on the average of four images taken for each condition with both filters. The effect of PHF6 on the number of dead cells/mm² was analyzed using t-test with a p-value ≤ 0.01.

3.5.5 *Lipid monolayer insertion assays*

A Langmuir trough (KSV Instruments Ltd., Finland) Micromini model with Delrin barriers was used to measure insertion of NH₃⁺-PHF6 and Ac-PHF6 into a lipid monolayer composed of 100% DMPG. Water (50 mL) was used as the subphase, and experiments were performed at 30 ± 0.5 °C (temperature was controlled via a circulating water bath). DMPG was initially dissolved at 3.0 mg/mL in 7:3 v/v chloroform to methanol solution and sonicated. TR-DHPE was dissolved in 9:1 chloroform to methanol at 0.5 mg/mL. The final spreading solution for the trough was composed of 0.2 mg/mL DMPG with 0.5 mol% TR-DHPE in 9:1 v/v chloroform to methanol. After spreading the lipids on the air/water interface, solvent was
allowed to evaporate for 10 minutes before compressing the lipids with symmetric barriers at 3.0 mm/sec to a surface pressure of 25 mN/m. For constant area experiments, the barrier position was fixed so that peptide insertion resulted in pressure increase. NH$_3^+$-PHF6 and Ac-PHF6 were dissolved in water at 800 μM, vortexed, and the supernatant was collected after 10 minutes of centrifugation at 14,000 rpm to remove insoluble peptides. Peptides were injected into the subphase to reach a final concentration of 5 μM in the trough subphase. Fluorescence microscopy images were taken with an IX51 model inverted fluorescent microscope (Olympus) with an Exi Aqua Bio-Imaging Camera (QImaging).

3.5.6 X-ray scattering experiments

X-ray scattering experiments were performed at the Advanced Photon Source at Argonne National Labs, Sector 15 NSF’s ChemMatCARS. Experiments were carried out in a 6.5x6.5 cm$^2$ (20 mL subphase volume) Langmuir trough equipped with a Wilhelmy plate balance. Water subphase was degassed and maintained at room temperature (~23˚C) and DMPG was deposited at the air/water interface to form a monolayer at 25 mN/m. An aliquot of NH$_3^+$-PHF6 or Ac-PHF6 was then injected into the subphase to a final peptide concentration of 5 μM in the trough. To prevent background scattering and oxidative beam damage to the monolayer, the trough was in a sealed canister and purged with helium gas to reduce the gaseous oxygen content to < 2%. The wavelength of the X-ray beam was 1.24 Å, and X-rays were detected with a Dectris PILATUS 100K detector. Data was integrated using Python software built by beamline support scientists (https://github.com/weibu/Liquid_Surface_ChemMatCARS). A typical set of XR and GIXD scans required approximately 45-60 min to obtain. As an additional precaution against beam damage, the trough was systematically translated during data collection. The dimensions of the incoming X-ray beam footprint on the liquid surface
were ~1 mm by 3-10 mm for XR, and ~1 mm by 29 mm for GIXD. X-ray reflectivity (XR) and GIXD measurements were collected and analyzed as described in the Supplemental Material.

3.5.7 Preparation of large unilamellar vesicles (LUVs)
LUVs composed of 70 mol% POPC and 30 mol% POPG were prepared as previously described\textsuperscript{31}. A 4 mM lipid solution was prepared in chloroform and dried overnight under vacuum. Dried lipid film was rehydrated with MilliQ water to 2 mM concentration. Lipid solution was vortexed and incubated for 30 minutes at room temperature before being exposed to five freeze/thaw cycles. Finally, rehydrated lipids were extruded 19 times using an extruder with 100 nm pore size polycarbonate membranes (Avanti Polar Lipids, Alabaster, AL).

3.5.8 Circular dichroism spectroscopy
PHF6 secondary structures were analyzed immediately after solubilization in water at 168 µg/mL and after 1 hour incubation in the presence of 688 µM LUVs by circular dichroism (CD) spectroscopy using an AVIV 410 CD spectrometer (Aviv Biomedical Inc., Lakewood, NJ). A 0.1 cm path length quartz cell (Starna Cells, Atascaders, CA) was used and CD spectra were obtained in triplicates between 180-260 nm with an averaging time of 15 s. The average CD signal in millidegree was then converted to molar ellipticity ($\theta$)\textsuperscript{119}.

3.5.9 Dynamic light scattering (DLS)
LUVs were analyzed by DLS to determine their size distribution when incubated alone at 20 µM or in the presence of 50 µg/mL NH$_3$\textsuperscript{+}-PHF6 or Ac-PHF6. Samples were analyzed using a DAWN HELEOS II light scattering instrument (Wyatt Technology Corporation, Santa Barbara, CA) in a quartz microcuvette. Scattering intensity at 90° was recorded over 10 min and analyzed using the regularization tool on ASTRA 5.3.4.20 software.
3.5.10 Transmission electron microscopy (TEM) imaging

PHF6 peptides were imaged by TEM to visualize fibrillation. Peptides were diluted to 50 μg/mL with water and 4 μL of the diluted sample was loaded onto a glow discharge (30 s) copper grid. Excess sample was wicked away and the grid was stained with 2 % uranyl acetate 4 times, 4 μL of stain for 5 min followed by 3 more stains of 1 min each. Between each staining step, the excess of stained was wicked away. After staining, the grid was air dried and was imaged using HITACHI HT7700 transmission electron microscope (Hitachi High Technologies Corp., Tokyo, Japan).

3.6 Acknowledgements

X-ray scattering experiments were performed at the Advanced Photon Source at Argonne National Laboratory using NSF’s ChemMatCARS Sector 15 beamline. NSF’s ChemMatCARS Sector 15 is supported by the Divisions of Chemistry (CHE) and Materials Research (DMR), National Science Foundation, under grant number NSF/CHE-1834750. Use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357. We are thankful to Dr. James Stroud and Taylor Britton who originally synthesized both NH$_3^+$-PHF6 and Ac-PHF6. We also express our gratitude to Dr. Erik B. Watkins for helpful discussion and review of this manuscript.

3.7 Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.
3.8 Footnote

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Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

3.9 Supplementary Information

3.9.1 Supplemental Experimental Procedure: X-ray scattering data analysis

X-ray reflectivity (XR) was used to measure the electron density of materials deposited at the air/water interface on a Langmuir trough. X-ray scattering theory and the liquid diffractometer used here have been fully described previously\textsuperscript{120-122} so they are only briefly summarized here. By measuring the intensity of reflected X-rays, one can deduce detailed information on the electron density distribution normal to the interface, \( \rho(z) \), laterally averaged over both the ordered and disordered parts of the film. The reflectivity is defined as the ratio of reflected to incident beam intensities, in a specular geometry, as a function of the vertical momentum transfer vector \( q_z = (4\pi/\lambda) \sin \theta \), where \( \theta \) is the incident angle of the X-ray on the surface. Finally, the reflectivity curve can be analyzed to obtain the in-plane averaged electron density distribution normal to the interface.

The data was collected by tilting a germanium monochromator crystal to deflect the beam and change the angle of incidence on the sample. Intensities were collected over the
range 0.01 < \( q_z < 0.8 \) Å\(^{-1}\), background subtracted, and normalized to incident beam flux. Data presented are divided by the Fresnel reflectivity (scattering from infinitely sharp air-water interface) \((R_F)\) with error bars representing one standard deviation error for each data point. Division by the Fresnel reflectivity provided better visualization of the XR data. In fitting reflectivity data, a ‘slab’ model was used to obtain the electron density profile normal to the interface\(^{31}\). The studied system was divided into layers, or slabs, of certain thickness, constant \( \rho \) and interconnected by interfacial roughness approximated by error functions. The parameters of such model were adjusted using Motofit program\(^{123}\) to obtain lowest \( \chi^2 \) values and reasonable values of the parameters.

In GIXD experiments, an evanescent wave was generated by an incident beam striking the water surface at an angle corresponding to a value of momentum transfer vector in the \( z \) direction, \( q_z = 0.85q_c \) where \( q_c = 0.02176 \) Å\(^{-1}\) is the critical momentum transfer vector for total external reflection from the water subphase. Diffraeted intensities were recorded as a function of both \( q_z = 2\pi\sin\alpha_f/\lambda \) where \( \alpha_f \) is the out-of-plane angle of the diffracted beam, and \( q_{xy} \approx 4\pi\sin(\theta_{xy}/2)/\lambda \) where \( \theta_{xy} \) is the angle between the incident and diffracted beam projected onto the horizontal plane. The GIXD intensity resulting from a powder of 2-D, azimuthally disoriented, crystallites can be represented as Bragg peaks (resolved in the \( q_{xy} \) direction but integrated over the \( q_z \)) or Bragg rods (resolved in the \( q_z \) direction but integrated over the \( q_{xy} \)). The scattered signal was recorded using a 2-D Pilatus 100K PSD and slits to define a pinhole focusing geometry to yield a \( q_z \) vs. \( q_{xy} \) intensity distribution with a horizontal resolution of \( \Delta q_{xy} = 0.006 \) Å\(^{-1}\). GIXD data were background subtracted and the Bragg peaks were fit with the Multi Peak Fit 2 function for Igor with Gaussian, Lorentzian, or Voigt profiles depending on the goodness of fit. A conservative 15 % error is assumed for the full width half maximum
(FWHM) values from Voigt profile fits. The $q_{xy}$ resolution of the ChemMatCARS liquid surface instrument of $\Delta q_{xy} = 0.006 \text{ Å}^{-1}$ was taken into consideration to calculate the FHHM values. The angular positions of the Bragg peaks determine the $d$-spacings, $d = 2\pi / q_{xy}^{\text{max}}$ (where the $q_{xy}^{\text{max}}$ is the position of the maximum of the Bragg peak) for the 2D lattice. For hexagonally packed phospholipid acyl tails, intermolecular distance does not correspond directly to $d$-spacing, but is calculated from the $d$-spacing by the trigonometric relationship $\text{intermolecular distance} = d$-spacing / $\sin(60)$. From the FWHM of the peaks, coherence length $L_c$ of the 2D crystallites, the average distance in the direction of the reciprocal lattice vector $q_{xy}$ over which ordering extends, can be determined using Equation 2.

$$L_c = \frac{0.9 x 2\pi}{\sqrt{\text{FWHM}^2 - 0.006^2}}$$  

(2)

For analysis, the areas of the Bragg peaks indicate the amounts of diffracting material present at the air/water interface. The intensity distribution along the Bragg rod reflects the molecular structure factor and could be analyzed to determine the length of the coherently scattering moiety, $L_m$, magnitude of its tilt vis-à-vis the surface normal, and the magnitude of molecular motion. The FWHM in the Bragg rod intensity distribution relates to the length of the diffracting molecular entity. The Bragg rods measured in this work were fitted with Equation 2 to calculate $L_m$ values. Since the Bragg rods were very broad and the instrumental resolution along $q_z$ is small (limited only by the size of the detector’s pixel), Equation 2 was used without calculating intrinsic FWHM. Detailed analysis methods and theory have been previously described.$^{121, 122, 124}$
3.9.2 Supplementary Figures and Tables:

**Figure S1:** Surface activity measurements of NH$_3^+$-PHF6 and Ac-PHF6. Adsorption of 3 μM PHF6 to a clean air/water interface at 30 °C.

**Table S1:** Complete GIXD Fit Parameters for NH$_3^+$-PHF6

<table>
<thead>
<tr>
<th>Sample</th>
<th>$q_{xy}$ Position (Å$^{-1}$)</th>
<th>FWHM</th>
<th>d Spacing (Å)</th>
<th>Inter-Molecule Distance (Å)</th>
<th>Integrated Peak Area</th>
<th>Coherence Length $L_c$ (Å)</th>
<th>Surface Pressure (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPG</td>
<td>1.491 ± 0.007</td>
<td>0.0124 ± 0.0004</td>
<td>4.213 ± 0.017</td>
<td>4.87 ± 0.02</td>
<td>668 ± 12</td>
<td>513 ± 15</td>
<td>25.2</td>
</tr>
<tr>
<td>NH$_3^+$-PHF6 – 1.0 h</td>
<td>1.462 ± 0.007</td>
<td>0.0492 ± 0.003</td>
<td>4.30 ± 0.02</td>
<td>4.96 ± 0.02</td>
<td>820 ±50</td>
<td>115 ± 6</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>1.498 ± 0.007</td>
<td>0.0107 ± 0.0003</td>
<td>4.194 ± 0.017</td>
<td>4.84 ± 0.02</td>
<td>690 ± 20</td>
<td>628 ± 18</td>
<td></td>
</tr>
<tr>
<td>NH$_3^+$-PHF6 – 3.5 h</td>
<td>1.497 ± 0.007</td>
<td>0.0114 ± 0.0004</td>
<td>4.196 ± 0.018</td>
<td>4.85 ± 0.02</td>
<td>277 ± 9</td>
<td>580 ± 20</td>
<td>31.7</td>
</tr>
<tr>
<td>NH$_3^+$-PHF6 – 5.0 h</td>
<td>1.499 ± 0.007</td>
<td>0.0111 ± 0.0004</td>
<td>4.191 ± 0.018</td>
<td>4.84 ± 0.02</td>
<td>246 ± 8</td>
<td>600 ± 20</td>
<td>30.8</td>
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<tr>
<td>NH$_3^+$-PHF6 – 7.0 h</td>
<td>1.498 ± 0.007</td>
<td>0.0113 ± 0.0003</td>
<td>4.194 ± 0.017</td>
<td>4.84 ± 0.02</td>
<td>310 ± 8</td>
<td>582 ± 18</td>
<td>28.6</td>
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**Table S2**: Complete GIXD Fit Parameters for Ac-PHF6. Inter-molecule distance refers to the distance between alkyl tails in case of diffraction from DMPG lipids.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( q_{xy} ) Position (( Å^{-1} ))</th>
<th>FWHM</th>
<th>( d ) Spacing - Unit Cell ( a, b ) (Å)</th>
<th>Inter-Molecule Distance (Å)</th>
<th>Integrated Peak Area</th>
<th>Coherence Length ( L_c ) (Å)</th>
<th>Surface Pressure (mN/m)</th>
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</thead>
<tbody>
<tr>
<td>DMPG</td>
<td>1.490 ± 0.007</td>
<td>0.012 ± 0.002</td>
<td>4.218 ± 0.018</td>
<td>4.870 ± 0.015</td>
<td>890 ± 40</td>
<td>480 ± 80</td>
<td>28.9</td>
</tr>
<tr>
<td>Ac-PHF6 – 1.0 h</td>
<td>1.477 ± 0.007</td>
<td>0.077 ± 0.011</td>
<td>4.25 ± 0.02</td>
<td>4.912 ± 0.017</td>
<td>1200 ± 100</td>
<td>73 ± 11</td>
<td>37.3</td>
</tr>
<tr>
<td>Ac-PHF6 – 3.5 h</td>
<td>1.461 ± 0.007</td>
<td>0.059 ± 0.009</td>
<td>4.30 ± 0.02</td>
<td>4.966 ± 0.018</td>
<td>1210 ± 60</td>
<td>95 ± 14</td>
<td>33.3</td>
</tr>
<tr>
<td>Ac-PHF6 – 5.0 h</td>
<td>1.481 ± 0.007</td>
<td>0.074 ± 0.011</td>
<td>4.24 ± 0.02</td>
<td>4.897 ± 0.017</td>
<td>860 ± 60</td>
<td>76 ± 11</td>
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<tr>
<td>Ac-PHF6 – 7.0 h</td>
<td>1.478 ± 0.009</td>
<td>0.062 ± 0.009</td>
<td>4.25 ± 0.03</td>
<td>4.910 ± 0.02</td>
<td>150 ± 50</td>
<td>90 ± 14</td>
<td>26.3</td>
</tr>
</tbody>
</table>

**Lipid Diffraction Peaks**

**Ac-PHF6 β-Sheet Diffraction Peaks**

**Ac-PHF6 low \( q_{xy} \), Protein Diffraction Peaks**

* A conservative 15% error is assumed for FWHM and \( L_c \) values.
**Figure S2:** 2D grazing incidence X-ray diffraction plots. Raw data used for grazing incidence X-ray diffraction. Color bar on the right indicates the scale of the images, with once scale used for the top DMPG sample, another scale used for all Ac-PHF6 samples, and a third scale used for all images on the bottom (second DMPG sample and all NH₃⁺-PHF6). Data collection artifacts besides the diffraction peaks are observed for Ac-PHF6 7 hours and NH₃⁺-PHF6 3.5 hours. In the first case the constant line of scattering at qₜ₉=1.1 Å⁻¹ was caused by an accidental vibration of the liquid surface. We believe that the scattering along Scherrer-ring observed in case of NH₃⁺-PHF6 at 3.5 hours was caused by a contaminating aggregate at the surface which came into the footprint of the X-ray beam – the Langmuir trough was moved occasionally perpendicular to the x-ray beam during the scans to avoid excessive beam damage. Returning to the same position in the trough at 9 hours produced the same Scherrer-ring, thus the signal is a position-dependent contaminant and not a time-dependent scattering feature.

**Table S3:** NH₃⁺-PHF6 XR Fit Parameters

<table>
<thead>
<tr>
<th></th>
<th>Slab 1 (Tails)</th>
<th>Slab 2 (Heads)</th>
<th>Slab 3 (outside layer)</th>
<th>Subphase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thickness</td>
<td>ρ / ρwater</td>
<td>Roughness</td>
<td>Thickness</td>
</tr>
<tr>
<td>DMPG</td>
<td>15.9 ±0.2</td>
<td>0.98 ±0.03</td>
<td>3.3 ±0.3</td>
<td>9.1 ±0.3</td>
</tr>
<tr>
<td>PHF6 2.5 h</td>
<td>16.5 ±0.3</td>
<td>1.019 ±0.004</td>
<td>3.52 ±0.03</td>
<td>8.9 ±0.5</td>
</tr>
<tr>
<td>PHF6 4.5 h</td>
<td>16.2 ±0.4</td>
<td>1.009 ±0.005</td>
<td>3.52 ±0.04</td>
<td>9.0 ±0.7</td>
</tr>
<tr>
<td>PHF6 6.5 h</td>
<td>16.0 ±0.3</td>
<td>1.017 ±0.007</td>
<td>3.29 ±0.06</td>
<td>9.3 ±0.5</td>
</tr>
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</table>
Table S4: Ac-PHF6 XR Fit Parameters

<table>
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<tr>
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<th>Slab 1 (Tails)</th>
<th>Slab 2 (Heads)</th>
<th>Slab 3 (outside layer)</th>
<th>Subphase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thickness</td>
<td>ρ / ρwater</td>
<td>Roughness</td>
<td>Thickness</td>
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<td>DMPO</td>
<td>15.7 ± 0.4</td>
<td>0.931 ± 0.007</td>
<td>3.59 ± 0.05</td>
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</tr>
<tr>
<td>2.5 h</td>
<td>7.2 ± 0.2</td>
<td>0.607 ± 0.005</td>
<td>12 *</td>
<td>13.77 ± 0.03</td>
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<tr>
<td>4.5 h</td>
<td>15.0 ± 0.1</td>
<td>0.805 ± 0.003</td>
<td>2.3 *</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>6.5 h</td>
<td>4.7 ± 0.2</td>
<td>0.746 ± 0.005</td>
<td>13.1 *</td>
<td>13.54 ± 0.04</td>
</tr>
</tbody>
</table>

*A Fixed to reduce number of parameters during fitting

Figure S3: Model for PHF6 fibril structure deduced from published cryo-EM structures of Tau filaments. (A) Structure of 306-VQIVYK-311 (blue) and interacting partner 373-THKLTF-378 (green) are extracted from the complete structure of a paired helical filament isolated from an Alzheimer’s disease brain (PDB 5O31). VQIVYK forms parallel β-sheets with cross-sheet interactions with sidechains of THKLTF. (B) Atomistic structure of 306-VQIVYK-311 (blue) interactions with 373-THKLTF-378 (green). The two peptides form a hydrophobic core of amino acid side chains to stabilize interactions cross β-sheet interactions. (C) Predicted structure for PHF6 fibrils assuming a similar cross β-sheet packing motif as the natural paired helical filament isolated from Alzheimer’s disease brain. The original VQIVYK (blue) remains in position, while a second copy of VQIVYK (pink) was rotated and translated from the original coordinates to optimize the hydrophobic core of the structure.
**Figure S4:** Bragg rod analysis of the protein diffraction peak centered at 1.328 Å⁻¹ measured for Ac-PHF6 interactions with DMPG 5 hours after protein injection. Bragg rod data was extracted by integrating X-ray diffraction images along $q_z$ for the span of the diffraction peak. Data was fit with a Lorentzian model centered around 0.08 Å⁻¹ with an amplitude of 497 ± 7. The resulting FWHM from the fit was 0.277 ± 0.008 Å⁻¹, corresponding to a calculated $L_m$ value of 20.2 ± 0.6 Å. The position of the maximum of the Bragg rod at 0.08 Å⁻¹ above the horizon (defined as $q_z=0$ Å⁻¹) testifies that the coherently scattering molecular entity is slightly tilted from the interface normal. The sharp intensity spike at $q_z\sim 0$ Å⁻¹ is the Vineyard-Yoneda peak resulting from interference of the incident and refracted beams.

**Figure S5:** Size distribution of LUVs (20 µM lipids, 70%mol POPC, 30%mol POPG) incubated at room temperature over time. LUV are characterized by an overall size of 50 nm. Over 7 days of incubation at room temperature, the LUV overall size distribution was unchanged indicating high stability.
Chapter 4: High Selectivity and Sensitivity of Oligomeric p-Phenylene Ethynlenes for Detecting Fibrillar and Pre-fibrillar Amyloid Protein Aggregates

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\textsuperscript{1}Center for Biomedical Engineering, \textsuperscript{2}Biomedical Engineering Graduate Program, \textsuperscript{3}Department of Chemical and Biological Engineering, \textsuperscript{4}Department of Molecular Genetics and Microbiology, \textsuperscript{5}Department of Neurology, University of New Mexico, Albuquerque, New Mexico 87131, United States

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\textbf{Figure 4-1:} TOC graphic
4.1 Abstract

Misfolding and aggregation of amyloid proteins into fibrillar aggregates is a central pathogenic event in neurodegenerative disorders such as Alzheimer’s (AD) and Parkinson’s diseases (PD). Currently, there is a lack of reliable sensors for detecting the range of protein aggregates involved in disease etiology, particularly the pre-fibrillar aggregate conformations that are more neurotoxic. In this study, the fluorescent sensing of two novel oligomeric $p$-phenylene ethynylenes (OPEs), anionic OPE1- and cationic OPE2+, for detecting pre-fibrillar and fibrillar aggregates of AD-associated amyloid-$\beta$ (A\textbeta 40 and A\textbeta 42) and PD-associated $\alpha$-synuclein proteins (wildtype, and single mutants A30P, E35K, and A53T) over their monomeric counterparts, were tested. Furthermore, the performance of OPEs was evaluated and compared to thioflavin T (ThT), the most widely used fibril dye. Our results show that OPE1- and OPE2+ exhibited aggregate-specific binding inducing large fluorescence turn-on and spectral shifts based on a combination of backbone planarization, hydrophobic unquenching, and super-luminescent OPE complex formation sensing modes. OPEs exhibited higher selectivity, higher binding affinity, and comparable limits of detection for A\textbeta 40 fibrils compared to ThT. OPE2+ exhibited the largest fluorescence turn-on and highest sensitivity. Significantly, OPEs detected pre-fibrillar aggregates of A\textbeta 42 and $\alpha$-synuclein that ThT failed to detect. The superior sensing performance, the non-protein specific detection, and the ability to selectively detect fibrillar and pre-fibrillar amyloid protein aggregates point to the potential of OPEs to overcome the limitations of existing probes and promise significant advancement in the detection of the myriad of protein aggregates involved in the early stages of AD and PD.

Keywords: Alzheimer’s and Parkinson’s diseases, oligomers, amyloid fibrils, protein aggregate detection, fluorescent optical probes, oligomeric $p$-phenylene ethynylenes
4.2 Introduction

Neurodegenerative disorders such as Alzheimer’s (AD) and Parkinson’s diseases (PD) are one of the biggest global health crises of the 21st century, with about 50 million people affected worldwide and at a cost that is projected to rise to $2 trillion per year by 2030 in the United States. AD and PD are complex and multifactorial. While many factors may contribute to disease etiology, including inflammation and microbial infection, the misfolding and aggregation of largely intrinsically disordered proteins into highly ordered fibrillar amyloid aggregates is still recognized as a central pathogenic event and one that is believed to occur decades before the onset of neurodegeneration and cognitive impairment. Monitoring the course of amyloid formation at the biochemical, cellular, and tissue levels is thus vital to understanding and combating these diseases.

Amyloid formation is complex and heterogeneous, and proceeds through the formation of a range of oligomeric intermediates that have been shown to be the primary toxic species that lead to neuronal loss and synaptic dysfunction. Thus, useful probes for tracking the disease process need to exhibit sufficient sensitivity to detect an array of aggregate conformations, including oligomers as well as fibrils. In addition, comorbidities among the diseases, for example, 80% of PD patients develop dementia, caused by the co-deposition of multiple pathological proteins necessitates that useful probes simultaneously detect aggregates formed by different proteins. For instance, Alzheimer’s disease is characterized by the accumulation of both amyloid plaques composed of the amyloid-β (Aβ) peptide and neurofibrillary tangles made of the tau protein. In the case of Parkinson’s disease, the deposition of Lewy bodies composed of the α-synuclein protein are observed as well as the accumulation of tau aggregates. Probes that can detect aggregates made of a variety of
proteins are therefore potentially clinically useful. Unfortunately, no such probes are currently available.

Many studies have sought to develop probes for protein aggregates, including conformation- and sequence-specific antibodies\textsuperscript{154}. However, the myriad of challenges associated with antibody-based detection and failures of immunotherapy clinical trials\textsuperscript{155, 156} have spurred renewed efforts in developing small-molecule probes for detecting and localizing amyloids. Fluorescent dyes\textsuperscript{157}, radiolabeled positron-emission tomography (PET), single photon emission computed tomography (SPECT) ligands\textsuperscript{158-161}, and metal-ligated magnetic resonance imaging (MRI) contrast agents\textsuperscript{162} have been developed but their diagnostic value is thus far limited. For example, Pittsburgh Compound B\textsuperscript{158} and $^{18}$F-AV-45\textsuperscript{159} only detect amyloid-β (Aβ) fibrillar deposits in the AD brain. They are of limited use because they fail to detect neurotoxic pre-fibrillar aggregates that better correlate with clinical symptoms. They also do not detect tau aggregates, further undermining their diagnostic value as AD is characterized by complex regional localization of both Aβ and tau deposits. Additionally, no clinically tested probes are available for imaging PD-related α-synuclein aggregates\textsuperscript{163}.

Compounds that bind specifically to amyloids tend to share a common “rigid conjugated rod” motif, where the linear shape and aromaticity favors binding to hydrophobic sites on the amyloid fibril\textsuperscript{164, 165}. Probes that have been investigated include molecules derived from Congo red, curcumin\textsuperscript{166-168}, thioflavin T (ThT)\textsuperscript{169-171}, and oligo-thiophenes\textsuperscript{172-177}. These ligands, when used for fluorescent imaging, primarily function on a “molecular rotor” basis, in which fluorescence enhancement and red shift arise from backbone planarization (restriction to rotation) of the conjugated region within the fluorophore when bound to a planar site on the
amyloid fibril. This single sensing mode gives rise to the probes’ ability to detect primarily the fibrillar conformation of amyloid proteins.

Recently, we tested a novel class of fluorescent sensors, oligomeric p-phenylene ethynylenes (OPEs) for the selective detection of fibrillar aggregates of model proteins over their monomeric counterparts\textsuperscript{81, 82}. The phenylene ethynylenes (PE) backbone of the OPEs has a delocalized electronic structure, which gives rise to strong absorption and emission\textsuperscript{178-180}. Importantly, the rapid transport of the electronic excited state along the backbone contributes to increased sensitivity, leading to signal amplification when interacting with analytes\textsuperscript{178-180}. Water soluble PE-compounds suitable for biological applications such as OPEs have shown greater than $10^6$-fold amplification sensitivity to quenching, that is, the probes are quenched in water through partial proton transfer from an interfacial water molecule to the OPE terminal oxygen causing rapid deactivation of the excited singlet state, and exhibit fluorescence “turn-on” in response to release of interfacial water, changes in their conformation, and/or ligand interactions\textsuperscript{83, 178, 181, 182}. In addition to backbone planarization induced fluorescence increase\textsuperscript{181}, bathochromic shift, hydrophobic unquenching (e.g., from binding to a hydrophobic site) and formation of super-luminescent OPE-complexes offer additional fluorescence “turn-on” sensing mechanisms\textsuperscript{81, 82, 87}.

OPEs have been shown to selectively bind to β-sheet enriched fibrillar aggregates made of the model amyloid protein hen egg white lysozyme (HEWL)\textsuperscript{81}. Upon binding, OPEs exhibited fluorescence turn-on and red shifted spectra; complexation of the OPEs to form J-dimers was also observed\textsuperscript{81, 82, 181}. A library of 18 PE-based oligomers and polymers that vary in size, charge, water solubility, and the types and geometries of functional groups, was screened for their capability to selectively detect the amyloid conformation of two model
proteins that are oppositely charged at physiological pH, bovine insulin and HEWL. We found that characteristics contributing toward fibril-selective, but not protein-specific detection included moderate size, negative charge, and high environmental sensitivity of the ethyl ester termini to fluorescence quenching in water. One compound in particular, the anionic OPE1- (Table 4-1), was found to show high binding selectivity toward both insulin and HEWL, and the cationic OPE2+ (Table 4-1), although displayed stronger fluorescence enhancement in the presence of HELW fibrils, did not detect insulin fibrils.

To evaluate the efficacy of the OPEs for detecting disease-relevant protein aggregates, we evaluated in this study the capability of OPE1- and OPE2+ to selectively detect fibrillar and pre-fibrillar aggregates of AD associated Aβ40 and Aβ42 peptides and four variants of PD associated α-synuclein proteins over their monomeric counterparts. First, fibrillar and pre-fibrillar aggregates of the proteins were prepared and characterized. Next, selectivity and specificity of OPE1- and OPE2+ to detect the aggregated conformation of the proteins were quantified and compared to ThT.

4.3 Results and Discussion

In this study, we tested the sensing capability of two ethyl ester-terminated OPEs, the negatively charged OPE1- characterized by one repeat unit and two side chains each terminated with a sulfonate group and the positively charged OPE2+ with two repeat units and two side chains each terminated with a quaternary amine group (Table 4-1). OPE sensing of Aβ40 and Aβ42 fibrils associated with AD as well as protofibrils and fibrils made of four different α-synuclein proteins involved in PD was evaluated. Additionally, performance of the OPE sensors was compared to the most commonly used amyloid dye ThT (Table 4-1).
**Table 4-1: Structures of thioflavin T, OPE1- and OPE2+**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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<tbody>
<tr>
<td>Thioflavin T</td>
<td><img src="image" alt="Thioflavin T structure" /></td>
</tr>
<tr>
<td>OPE1-</td>
<td><img src="image" alt="OPE1- structure" /></td>
</tr>
<tr>
<td>OPE2+</td>
<td><img src="image" alt="OPE2+ structure" /></td>
</tr>
</tbody>
</table>

### 4.3.1 Formation and characterization of Aβ40 fibrils

Aβ40 fibrils are known to display high polymorphism at the molecular level where the morphology and structure of the fibrils are highly sensitive to incubation conditions. A useful sensor needs to robustly detect the common fibrillar conformation even if their structures differ at the molecular scale. In this study, we tested OPE1- and OPE2+ detection of Aβ40 fibrils produced in two different conditions: Tris buffer at pH 8.0 (protein net charge: -4.4) and phosphate buffer (PB) at pH 7.4 (protein net charge: -2.9). The morphology and secondary structures of Aβ40 fibrils formed after 21 days of incubation at 37 °C and 100 μM were analyzed by TEM imaging and circular dichroism (CD) spectroscopy, respectively. Kinetics of Aβ40 fibril formation was also monitored using the standard ThT fluorescence assay.
TEM images of unincubated Aβ40 samples (Figure 4-2 A and C) show very few features; the dark spots are likely artifacts of the TEM grid or sample preparation. Size-exclusion higher performance liquid chromatogram (SEC) of the unincubated sample shows a single well-defined protein peak (see Figure S1 in Supplemental Information) corresponding to a low molecular weight species. Thus, Aβ in the unincubated sample was soluble and likely monomeric. TEM images of Aβ40 fibrils produced in pH 8.0 Tris (Figure 4-2 B) and in pH 7.4 PB (Figure 4-2 D) show twisted fibrils with similar widths (Tris: 11.5 ± 2.8 nm; PB: 13.3 ± 1.5 nm). However, the fibrils produced in Tris buffer appear to be longer (766 ± 250 nm) than those produced in PB buffer (261 ± 150 nm). Fibril samples were also centrifuged to remove insoluble aggregates and the supernatant was analyzed by SEC. Results show that 8% and 21% of monomers were left after 21 days of incubation in Tris and PB, respectively, and no oligomeric aggregates were present (Figure S1).

**Figure 4-2:** TEM images of unincubated Aβ40 (A and C) and after 21 days of incubation at 37 °C and 100 µM (B and D) in pH 8.0 Tris (A and B) or in pH 7.4 PB (C and D). Scale bars represent 200 nm.
The secondary structures of unincubated Aβ40 and fibrils were analyzed using CD (Figure 4-3 and Figure S2 in Supplemental Information). CD spectra show a well-defined peak at 200 nm for unincubated Aβ40, which is characteristic of random coils (Figure S2). Fibrils produced in both Tris and PB showed structures rich in β-sheets as evidenced by the presence of the peak at 218 nm (Figure 4-3).

![CD spectra](image)

**Figure 4-3:** Molar ellipticity of Aβ40 after 21 days of incubation in Tris pH 8.0 and PB pH 7.4 at 37 °C. The peak at 218 nm arises from β-sheet structures.

Fibril formation was additionally confirmed and tracked by the standard ThT fluorescence assay (Figure 4-4). Aβ40 samples incubated at different times were mixed with ThT at final concentrations of 5 µM Aβ and 20 µM ThT and the emission spectra of ThT were recorded at an excitation wavelength of 440 nm. Integrated emission intensities were normalized to that of mature fibrils (Figure 4-4). ThT fluorescence profiles of Aβ incubated in the two buffers were remarkably similar and showed characteristic features of nucleation-controlled aggregation kinetics, where a lag phase of about 7 days was followed by a rapid fibril growth phase and finally a plateau phase during which most of the monomeric proteins
had been converted to fibrils. Taken together, Aβ40 fibrils rich in β-sheets were successfully prepared by incubating the peptide in two different solution conditions. The fibrils were morphologically similar but appear to differ in lengths. Molecular-level structural differences, for example, parallel vs. anti-parallel β-sheet architectures\textsuperscript{188-190}, may exist between the fibrils. However, they were not characterized further using high-resolution methods in this study.

**Figure 4-4:** Fluorescence profiles of ThT (20 µM) and OPE (1 µM) mixed with Aβ40 (5 µM) incubated in Tris pH 8.0 (A) or PB pH 7.4 (B) for different days. Integrated fluorescence intensities were background subtracted and normalized to signal obtained from samples incubated for 21 days. Errors bars are standard deviations from triplicate samples.
4.3.2 Fluorescence of OPE binding to Aβ40 fibrils and monomers

To assess OPE binding to Aβ40 fibrils, fluorescence of each OPE mixed with Aβ samples incubated for different times was measured (Figure 4-4) at a low concentration of 1 µM OPE. As shown in Figure 4-4, normalized fluorescence intensities of the two OPEs yielded similar profiles compared to those obtained from ThT, indicating that the OPEs selectively detected Aβ40 fibrils as well as ThT at a higher concentration of 20 µM.

To further investigate the photophysical properties of OPE sensing, emission and excitation spectra of ThT, OPE1- and OPE2+ in the presence of unincubated and fibrillar Aβ40 at 5 µM protein and 1 µM sensor were analyzed (Figure 4-5). The integrated emission intensities were also calculated and statistically analyzed (Figure S4). Absorbance spectra were additionally recorded and shown in Figure S3.

Both OPEs are highly quenched in buffers showing low fluorescence intensities (black spectra in Figure 4-5). No significant fluorescence increases were observed in the presence of Aβ40 monomers over the OPE background, except for OPE2+ with Aβ monomers (blue spectra in Figure 4-5A3 and B3). These small increases in OPE2+ fluorescence could be due to weak electrostatic interactions between the negatively charged peptide and the positively charged OPE. In contrast, large fluorescence enhancements were observed when OPEs were mixed with Aβ40 fibrils produced in both Tris and PB. Additionally, red shifts in the excitation spectra were observed for OPE1- and OPE2+ (Table 4-2); blue shifts, albeit smaller, were also observed in emission spectra (Table 4-2 and Figure 4-5). Bathochromic red shifts can arise from OPE J-dimer formation driven by stacking interactions in the presence of two side chains located on opposite sides of the central phenyl ring 191, and/or planarization during complex formation, and/or desolvation of the terminal ethyl ester groups 192. This result is consistent
with the binding patterns of OPE1- and OPE2+ to HEWL and insulin fibrils previously reported\textsuperscript{81, 82}. In contrast, ThT at 1 µM exhibited small increases in fluorescence intensity in the presence of both fibrils (10 to 30-fold lower by comparison with OPEs fluorescence).

**Figure 4-5:** Excitation (dashed lines) and emission spectra (solid lines) of ThT (A1 and B1), OPE1- (A2 and B2), or OPE2+ (A3 and B3) alone (black) in the presence of Aβ40 monomers (blue) or Aβ40 fibrils (red) incubated in Tris pH 8.0 (A) and PB pH 7.4 (B). Fluorescence was recorded in the presence of 5 µM protein and 1 µM sensor.

**Table 4-2:** Maximum excitation and emission wavelengths of 1 µM ThT, OPE1- or OPE2+ sensors in the presence of 5 µM Aβ40 monomers or fibrils

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Fibril incubation condition</th>
<th>Max excitation/emission wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensor alone</td>
</tr>
<tr>
<td>ThT</td>
<td>Tris pH 8.0</td>
<td>409/460</td>
</tr>
<tr>
<td></td>
<td>PB pH 7.4</td>
<td>412/459</td>
</tr>
<tr>
<td>OPE1-</td>
<td>Tris pH 8.0</td>
<td>372/477</td>
</tr>
<tr>
<td></td>
<td>PB pH 7.4</td>
<td>378/470</td>
</tr>
<tr>
<td>OPE2+</td>
<td>Tris pH 8.0</td>
<td>400/464</td>
</tr>
<tr>
<td></td>
<td>PB pH 7.4</td>
<td>402/474</td>
</tr>
</tbody>
</table>
4.3.3 Quantitative evaluation of sensor performance

Effective sensors of amyloid protein aggregates need to have high selectivity that detects the aggregated conformations but not their non-aggregated conformers, high sensitivity, high signal to background ratio, and high affinity. Sensors additionally need to be non-protein specific, that is, selective for the aggregate conformations of a range of protein protomers.

Our results show that OPE1- and OPE2+ bind specifically and selectively to Aβ40 fibrils over Aβ40 monomers at a concentration significantly lower than that usually used for ThT and induce 10 to 30-folds higher fluorescence intensity than ThT (Figure 4-5 A1 and B1). To quantitatively assess sensor performance, selectivity of OPEs toward Aβ40 fibrils at a 5 to 1 protein to sensor molar ratio was analyzed. The amyloid detection factor (ADF) was calculated using Equation 1 \(^82\), where \(F_{(\text{dye in buffer})}\), \(F_{(\text{dye+fibrils})}\) and \(F_{(\text{dye+monomers})}\) are the integrated emission intensities for sensor alone, sensor in the presence of fibrils, and sensor in the presence of monomers, respectively.

\[
ADF = \frac{F_{(\text{dye+fibrils})} - F_{(\text{dye+monomers})}}{F_{(\text{dye in buffer})}}
\]  \hspace{1cm} (1)

Positive ADF values indicate selective sensing of fibrils over monomers, while negative ADF values indicate selective sensing of the monomers. ADF values close to zero indicate no selective sensing, which for the ethyl ester terminated OPEs could be due to a lack of binding to either the monomeric or the fibrillar conformation of the protein. As shown in Table 3, all three sensors yielded positive ADF values for Aβ40 sensing, indicating that they are all selective sensors of Aβ40 fibrils over Aβ40 monomers. ThT was the least selective with an ADF value of 0.53 ± 0.1 that is closest to 0. The cationic OPE2+ was the most selective with the highest ADF values; an ADF value of 11 ± 1 was obtained for longer fibrils prepared in Tris buffer and an ADF value of 7.4 ± 0.5 was obtained for shorter fibrils prepared in PB buffer.
Selectivity of OPE1- (ADF values of around 2.5) was intermediate between ThT and OPE2+.

**Table 4-3:** Amyloid detection factor (ADF), limit of detection (LOD) and dissociation constant \( (K_d) \) for ThT, OPE1- and OPE2+ sensing of Aβ40 fibrils produced in pH 8.0 Tris and in pH 7.4 PB.

<table>
<thead>
<tr>
<th>Sensors</th>
<th>ADF ( \text{Aβ}40 ) in Tris</th>
<th>ADF ( \text{Aβ}40 ) in PB</th>
<th>LOD (µM) ( \text{Aβ}40 ) in Tris</th>
<th>LOD (µM) ( \text{Aβ}40 ) in PB</th>
<th>( K_d ) (µM) ( \text{Aβ}40 ) in Tris</th>
<th>( K_d ) (µM) ( \text{Aβ}40 ) in PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThT</td>
<td>0.53 ± 0.1</td>
<td>0.23 ± 0.03</td>
<td>0.65 ± 0.05</td>
<td>0.69 ± 0.05</td>
<td>2.7 ± 1</td>
<td>5.1 ± 2</td>
</tr>
<tr>
<td>OPE1-</td>
<td>2.8 ± 0.6</td>
<td>2.3 ± 0.3</td>
<td>0.52 ± 0.04</td>
<td>0.48 ± 0.02</td>
<td>0.70 ± 0.1</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>OPE2+</td>
<td>11 ± 1</td>
<td>7.4 ± 0.5</td>
<td>0.45 ± 0.1</td>
<td>0.24 ± 0.03</td>
<td>0.16 ± 0.02</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

Errors reported are standard errors.

Sensitivity is another important performance metric of sensors. For amyloid sensors, sensitivity depends on binding affinity and the magnitude of induced fluorescence. To compare sensitivity of the three compounds for detecting Aβ fibrils, their limits of detection (LOD) were determined (Equation 2)\(^{193, 194}\), which indicates the lowest protein concentration that can be detected by each sensor with a high degree of certainty. Fluorescence of the sensors at various protein concentrations were determined and the protein concentration corresponding to LOD was calculated by using the slope of the signal vs. concentration correlation (Equation 2).

\[
Concentration \ LOD = \frac{3\sigma}{S} \quad (2)
\]

where \( \sigma \) is the standard deviation of the dye alone (blank) and \( S \) is the slope of the signal vs. concentration correlation (Figure S5). As shown in Table 4-3, LOD values are in the sub-micromolar range for all three sensors. LOD values of ThT are consistent with previously reported values \(^{195}\). OPEs and ThT display similar LOD values for both Aβ40 fibrils and both OPEs display lower LOD values compared to that of ThT.
Finally, the affinity of ThT, OPE1- and OPE2+ binding to Aβ40 fibrils was evaluated through the determination of the dissociation constant ($k_d$) (Table 4-3). Binding curves were obtained for the dyes in the presence of Aβ40 fibrils produced in Tris and PB buffers (Figure S6). OPEs can bind as single molecules or as complexes, particularly to planar binding sites on the fibril surface. Indeed, OPEs have been shown to form J-dimers upon binding to HEWL fibrils. As described previously, the ethyl ester terminated OPEs have multiple binding-induced unquenching modes, including backbone planarization, hydrophobic unquenching, and formation of super-luminescent OPE-complexes. Each mode contributes to fluorescence enhancement, and backbone planarization and OPE-complexation additionally result in spectral shifts. To account for potential OPE-complexation, binding curves were fitted with a Hill coefficient (h) (Figure S6). Fitted parameters are summarized in Figure S6 and Table 4-3. Values of h for ThT are close to 1, indicating independent binding of ThT to fibrils. h values for the OPEs were around 1.5, indicating moderate positive cooperativity binding, which could be contributed by OPE-complexation. Importantly, $k_d$ values for OPE1- and OPE2+ were in the sub-micromolar range (Table 4-3). These values were lower than that obtained from ThT, indicating that the OPEs exhibit higher affinity toward Aβ fibrils.

Taken together, both OPEs display higher selectivity and affinity than ThT toward Aβ40 fibrils when tested at 1 μM sensor concentration. While sensing parameters of ThT were similar between the fibrils produced in the two different buffer conditions, some variabilities in OPE sensing parameters were seen. OPE2+ exhibited higher selectivity for the longer fibrils prepared in Tris than the shorter fibrils prepared in PB. OPE1- exhibited higher binding affinity toward the shorter fibrils than the longer fibrils. To resolve the cause of these differences, additional investigations of OPE-fibril binding and fibril structures are needed.
4.3.4 OPE Sensing of Aβ42 oligomers and fibrils

In addition to high selectivity, sensitivity and affinity, useful amyloid sensors also need to be non-protein specific, that is, capable of sensing the common amyloid conformation of a range of proteins involved in neurodegenerative diseases. To this end, we evaluated the capability of OPE1- and OPE2+ to detect aggregates of two additional amyloid proteins: amyloid-β (1-42) (Aβ42) and α-synuclein. Aβ42 is the more amyloidogenic form of Aβ\(^{196}\) and α-synuclein forms the Lewy bodies found in the brains of PD patients\(^{18}\). The deposition of Aβ40 and Aβ42 in the brain as amyloid plaques is a pathological hallmark of AD\(^{197}\). Because the deposition of Aβ42 starts prior to Aβ40, these fibrils constitute a suitable target for testing OPEs sensing\(^{197-200}\).

Aβ42 fibrils were produced by incubating 45 µM peptide in pH 7.4 20 mM PB at room temperature for 72 h. Unincubated and incubated samples were imaged using TEM (Figure 4-6). In contrast to unincubated Aβ40 samples where very few features were seen (Figure 4-2 A and C), unincubated Aβ42 samples showed a large number of globular features of about 10.6 ± 1.9 nm in diameter (Figure 4-6 A). These are likely oligomeric aggregates from either the incomplete solubilization of the peptide or aggregates that were already formed due to the peptide’s high aggregation propensity\(^{201, 202}\). Incubated samples showed an abundance of long fibrils with characteristic twists (Figure 4-6 B). To further characterize the unincubated Aβ42 sample, the sample was centrifuged and the supernatant was analyzed by SEC (Figure S7) and dynamic light scattering (DLS). SEC chromatograms of unincubated Aβ42 show the presence of a large peak at 21 minutes that likely corresponds to monomeric Aβ42. However, this peak is broad and with shoulders at 17 and 20 minutes, indicating the presence of larger size species. Additionally, the hydrodynamic radius (R\(_h\)) of the unincubated Aβ42 sample measured by DLS was around 30 nm. Taken together, both results revealed the presence of soluble and
heterogeneous higher molecular weight species in the unincubated Aβ42 sample, or soluble oligomers. With incubation, the oligomer population first became enriched (blue chromatograph of 2.4 h incubated sample in Figure S7) before they are depleted with fibril formation, where only one smaller monomer peak was observed after 72 h incubation (red chromatograph in Figure S7). The $R_h$ value of the 72 h incubated sample, after centrifugation to remove insoluble aggregates, was around 3 nm, corroborating the loss of soluble oligomers with the formation of Aβ42 fibrils. Our results thus indicate that there were abundant oligomeric, pre-fibrillar aggregates present in the unincubated Aβ42 sample and that after incubation most of the soluble Aβ42 formed fibrils.

**Figure 4-6:** TEM images of unincubated (A) and incubated (B) Aβ42. Scale bars represent 200 nm.

Fluorescence spectra of ThT, OPE1- and OPE2+ were measured in the presence of unincubated (oligomeric) and incubated (fibrillar) Aβ42 at 5 µM peptide with either 1 µM OPE or 20 µM ThT (Figure 4-7). As shown in Figure 4-7, ThT and OPE1- exhibited small fluorescence increases with oligomeric Aβ42 and displayed large fluorescence increases in the presence of fibrils. Strikingly, OPE2+ showed large fluorescence enhancements with both pre-fibrillar and fibrillar aggregates of Aβ42. Thus, all three sensors effectively detected the fibrillar conformation of Aβ42. The cationic and larger OPE2+ additionally detected Aβ42 oligomers, making it potentially a more useful sensor for disease detection. The emission peak
of OPE2+ in the presence of Aβ42 oligomers is slightly red-shifted from that measured for the sensor alone (462 nm to 469 nm), and is blue shifted from its emission peak in the presence of Aβ42 fibrils (462 nm to 445 nm) (Table 4-4). These different spectral features indicate different modes of binding-induced fluorescence turn-on, which could be due to differences in binding sites (hydrophobic grooves vs. planar binding sites for example) that promote different photophysical changes in the bound OPE or OPE complexes. More detailed studies on dye-protein interactions will be needed to resolve the different dye binding modes. Although the spectral difference of OPE2+ binding to oligomers vs. fibrils demonstrated here is not large (~25 nm), it does demonstrate the potential of OPEs to detect and distinguish between different conformational states of Aβ42 aggregates.

**Figure 4-7:** Excitation (dashed lines) and emission spectra (solid lines) of ThT (A1), OPE1- (A2) and OPE2+ (A3) alone (black), in the presence of Aβ42 monomers (blue) and Aβ42 fibrils (red). Fluorescence was recorded in the presence of 5 µM protein and 1 µM OPEs or 5 µM protein and 20 µM ThT.

**Table 4-4:** Maximum excitation and emission wavelengths of ThT, OPE1- and OPE2+ sensors in the presence of Aβ42 unincubated and fibrils

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Max excitation/emission wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensor alone</td>
</tr>
<tr>
<td>ThT</td>
<td>413/481</td>
</tr>
<tr>
<td>OPE1-</td>
<td>392/450</td>
</tr>
<tr>
<td>OPE2+</td>
<td>400/462</td>
</tr>
</tbody>
</table>
4.3.5 OPE sensing α-synuclein aggregates

The main pathological hallmark of Parkinson’s disease is Lewy bodies composed of α-synuclein fibrils \(^{203, 204}\). However, oligomeric α-synuclein aggregates have been found to play a central role in Parkinson’s disease neurodegeneration including disrupting mitochondrial function, autophagy and lysosomal degradation, membrane homeostasis, endoplasmic reticulum function and synapses, and can induce inflammation \(^{17-20}\). Furthermore, oligomers can also propagate between neurons leading to cell-to-cell spreading of α-synuclein pathology \(^{17-19}\). In this study, the wild type (WT), as well as three single mutants, A30P and A53T involved in early onset of Parkinson’s disease \(^{203, 204}\), and E35K designed to produce small oligomers \(^{19, 204}\), were used to test OPE sensing. A30P has been shown to aggregate slower than WT \(^{205}\), while A53T has been reported to exhibit faster fibrillization \(^{206}\). To evaluate the efficacy of OPE sensing of early aggregates and mature fibrils, we evaluated the binding selectivity of both OPEs toward pre-fibrillar and fibrillar α-synuclein aggregates over monomers and compare them to ThT.

As shown by TEM images in Figure 4-8, except A53T, all other α-synuclein proteins appeared monomeric at day 0. After 6 days of incubation, pre-fibrillar aggregates (> 30 nm of diameter) were present, and after 16 days of incubation, mature fibrils were observed. In the case of the fast aggregating A53T, at day 0, the protein was already in an elongated aggregate, and after 6 days of incubation, fibrils were present. Emission and excitation spectra were recorded for ThT, OPE1- and OPE2+ in the presence of the four α-synuclein proteins at days 0, 6 and 16 (Figure 4-9) at 1 µM sensor and 5 µM protein concentrations. The ADF values of the three sensors were calculated (Table 4-5) and dyes that exhibited positive sensing (ADF > 1) are indicated in yellow on the TEM images.
In the presence of unincubated (day 0) WT, A30P and E35K proteins, fluorescence spectra close to background were obtained for all three sensors, indicating no sensing of monomers. In the presence of fibrils made from all four proteins (day 16), all three sensors displayed fluorescence enhancements. Similar to the sensing of Aβ40 and Aβ42 fibrils, OPEs exhibited larger fluorescence enhancements compared to ThT (2 to 20-fold higher for OPE1-, 6 to 100-fold higher for OPE2+). Although fluorescence enhancements were observed from ThT, ADF values lower than 1 were obtained in the presence of WT and E35K fibrils which indicates poor fibril selectivity, while both OPEs showed higher fibril selectivity of all α-synuclein proteins tested (ADF value > 1). Thus, compared to ThT, OPEs are superior sensors for α-synuclein fibrils and exhibit higher selectivity, higher fluorescence signal, and are more robust, that is, capable of sensing fibrils of all α-synuclein proteins tested.

Importantly, the capability of the three sensors to detect pre-fibrillar α-synuclein aggregates was tested. ThT was only able to detect A53T aggregates after 6 days of incubation (ADF > 1), while both OPEs detected pre-fibrillar aggregates of WT, E35K and A53T (Figure 4-9 and Table 4-5). No significant fluorescence enhancement was observed for the 6-day incubated A30P sample where some oligomers were observed by TEM (Figure 4-8 E). This could be due to the lower abundance of oligomers as A30P exhibits a slower aggregation rate (Figure S8). Finally, despite the high OPE fluorescence enhancements observed in the presence of both pre-fibrillar and fibrillar conformations of α-synuclein, no significant differences in the features of OPE spectra (for example, shifts in excitation or emission peaks) were seen between sensors bound to oligomers and fibrils. Thus, although OPEs can selectively detect both pre-fibrillar and fibrillar aggregates of α-synuclein, these sensors cannot distinguish between the different aggregate conformations.
Figure 4-8: TEM images of unincubated (A, D, G, J), 6-day (B, E, H and K) and 16-day incubated (D, F, I, L) α-synuclein. Four different isoforms of α-synuclein were imaged: wild type (A, B, C), A30P (D, E, F), E35K (G, H, I) and A53T (J, K, L). The name of the sensor capable to detect the different forms of α-synuclein appears in each TEM image in yellow (ADF > 1). The asterisk indicates that A53T at day 0 was not completely monomeric. Scale bar represents 200 nm.
Figure 4-9: Excitation (dashed lines) and emission spectra (solid lines) of ThT (A1, B1, C1 and D1), OPE1- (A2, B2, C2 and D2) and OPE2+ (A3, B3, C3 and D3) alone (black), in the presence of α-synuclein at day 0 (blue lines), day 6 (red) and day 16 (green) of incubation. Four isoforms of α-synuclein were evaluated: WT (A), A30P (B), E35K (C) and A53T (D).
Table 4-5: Amyloid detector factor (ADF) of ThT, OPE1- and OPE2+ in the presence of α-synuclein aggregates produced at day 6, and fibrils produced at day 16 of incubation.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Day 6</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>A30P</td>
</tr>
<tr>
<td>ThT</td>
<td>0.14±0.01</td>
<td>0.093±0.01</td>
</tr>
<tr>
<td>OPE1-</td>
<td>1.5±0.1</td>
<td>0.16±0.3</td>
</tr>
<tr>
<td>OPE2+</td>
<td>6.6±2</td>
<td>0.45±0.08</td>
</tr>
</tbody>
</table>

The ADF was measured for a molar ratio 1µM sensor to 5µM protein. Errors reported are standard errors.

* The ADF measured for A53T was calculated by using day 0 as monomeric protein whereas it is actually an oligomer-like species.

4.4 Conclusions

In this study, we investigated two novel PE-based sensors, OPE1- and OPE2+, for the selective detection of amyloid protein aggregates over monomers and compared their performance to the widely used ThT dye. Pre-fibrillar and fibrillar aggregates of six AD and PD associated amyloid proteins were tested, Aβ40, Aβ42, wildtype α-synuclein, and 3 α-synuclein mutants. Compared to ThT, OPE1- and OPE2+ are more selective sensors for Aβ40, Aβ42 and α-synuclein fibrils. OPEs displayed sub-µM binding constant and protein concentration limits of detection of Aβ40 fibrils, both of which are lower than those determined for ThT. OPE2+ exhibited the highest sensitivity and significantly detected pre-fibrillar Aβ42 and α-synuclein aggregates that ThT did not detect. OPE1- also selectively detected α-synuclein pre-fibrillar aggregates. ThT is known to detect fibrillar aggregates by intercalating into the β-strands in the β-sheets.43, 44 Due to the multiple binding-induced unquenching modes of OPEs, including backbone planarization, hydrophobic unquenching, and formation of super-luminescent OPE-complexes, our results show that OPEs bound to and detected a wider range of aggregate conformations compared to ThT. Although the exact nature of the binding mechanisms are unclear, planar features on fibril surface can serve as sites for OPE binding, which can lead to
backbone planarization induced fluorescence enhancement. Binding to pre-fibrillar aggregates, particularly those with increased surface hydrophobicity, could be mediated by hydrophobic interactions which result in hydrophobic unquenching. The superior sensing capability of OPEs demonstrated in this study point to the potential of these new sensors to overcome the two major limitations of current probes, the inability to detect a wide range of amyloid aggregate conformations, including the more disease relevant oligomeric, pre-fibrillar aggregates, and the inability to simultaneously detect aggregates of different proteins. Overcoming these limitations promises significant advancement in the detection of the myriad of protein aggregates involved in the early stages of AD and PD.

4.5 Experimental Methods

4.5.1 Materials

Synthetic amyloid β-40 (Aβ40) purified by reverse phase HPLC (purity > 95 %) was purchased from Peptide 2.0 (Chantilly, VA). The recombinant amyloid β-42 (Aβ42) and α-synuclein proteins were produced and purified as previously described. The plasmids coding for α-synuclein wild type (WT) and three mutants (A30P, E35K and A53T) were generously provided by Dr. Roland Riek (ETH Zurich) and the plasmid coding for Aβ42 was provided by Dr. Park (Chosun University, South Korea). Phosphate buffer (PB) was prepared with sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrous purchased from Thermo Fisher Scientific (Waltham, MA). Tris was purchased from BioRad (Hercules, CA). Sodium chloride (NaCl), ammonium hydroxide (NH₄OH) and dimethyl sulfoxide (DMSO) were acquired from EMD Millipore (Burlington, MA). 1,1,1,3,3,3 Hexafluoro-2-propanol (HFIP) was purchased from Sigma-Aldrich (St. Louis, MO). Copper grids, 400 mesh, covered
by a Formvar film and 5-10 mm carbon layer were purchased from Ted Pella (Redding, CA). 2% aqueous uranyl acetate was purchased from Electron Microscopy Sciences (Hatfield, PA). Thioflavin T (ThT) (MW: 318.85 g/mol, extinction coefficient at 412 nm (ε412nm) in water: 3.6 x 10^4 M^{-1}cm^{-1}) was acquired from ACROS Organics (Belgium). OPEs were synthesized and purified by previously published procedures \(^{181}\) (OPE1-: 724.79 g/mol and ε370 nm in water (3.92 ± 0.013) x 10^4 M^{-1}cm^{-1}; OPE2+: 1002.64 g/mol and ε378nm in water (8.29 ± 0.033) x 10^4 M^{-1}cm^{-1}).

4.5.2 Aβ40 aggregation

Synthetic Aβ40 (4,329 g/mol, ε_{280nm} = 1,490 M^{-1}cm^{-1}, pI = 5.5) \(^{208}\) was solubilized in DMSO at 50 mg/mL. The peptide was sonicated in a bath sonicator for 1 min and was centrifuged at 14,000 g for 15 min. The supernatant was collected and the peptide concentration was determined by measuring the absorbance at 280 nm with a Nanodrop (Thermo Fisher Scientific, Waltham, MA). Aβ40 was then stored in -70°C until use. Two incubations were set up. First the protein was diluted in 40 mM Tris pH 8.0 buffer containing 150 mM NaCl and 0.01 % w/v sodium azide \(^{65}\). Second, the protein was diluted in 50 mM PB pH 7.4 buffer containing 100 mM NaCl and 0.01 % w/v sodium azide \(^{186}\). For both incubations, the final protein concentration was 100 µM and the protein was incubated at 37 °C for 21 days under quiescent condition. Every 2 to 3 days a sample was collected for characterization.

4.5.3 Aβ42 aggregation

Purified and lyophilized Aβ42 was solubilized in 100 % HFIP at 1 mg/mL and was sonicated for 30 s. Then, the protein was dried under vacuum overnight and stored at -70 °C for up to 1 month. Aβ42 was solubilized in 0.1 % NH₄OH at 1 mg/mL (222 µM) and was then diluted to
45 µM in pH 7.4 20 mM PB. Protein was incubated at room temperature for three days under quiescent condition.

4.5.4 α-synuclein aggregation

Four isoforms of α-synuclein proteins were used: wild type (WT) and 3 single point mutants (A30P, E35K and A53T). The lyophilized α-synuclein protein was first solubilized to 300 µM in pH 7.4 10 mM PB, 300 mM NaCl and then incubated at 37 °C on a vertical rotor at 50 rpm for 16 days.

4.5.5 Fluorescence and absorbance measurements

ThT and OPEs fluorescence spectra in the presence of Aβ40 was measured with the PTI QuantaMaster 40 steady state spectrofluorometer (HORIBA Scientific, Edison, NJ). ThT was mixed with Aβ40 at a 4 to 1 molar ratio (20 µM ThT to 5 µM protein) or 1 to 5 molar ratio (1 µM ThT to 5 µM protein) in 10 mM PB. OPE1- and OPE2+ were mixed with the protein at a 1 to 5 molar ratio (1 µM OPEs to 5 µM protein) in 10 mM PB. After 30 minutes of incubation at room temperature in the dark, samples were transferred to a quartz cuvette (Starna cells Inc., Atascadero, CA) and the emission and excitation spectra were recorded. The emission spectra were obtained at excitation wavelengths 440, 390, and 414 nm, for ThT, OPE1- and OPE2+, respectively. The excitation spectra were recorded at emission wavelengths of 480, 450, and 470 nm for ThT, OPE1- and OPE2+, respectively. Experiments were performed in triplicates. Absorbance spectra were measured in a quartz cuvette (PerkinElmer, Waltham, MA) on a Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, MA) at a molar ratio of 1 to 5 (1 µM sensor to 5 µM protein) between 250 nm – 700 nm.
### 4.5.6 TEM imaging

Aliquots of protein solutions were first diluted in Milli Q water to 5 μM and 4 μL of the diluted solution was added to a glow discharged (Harrick Plasma Cleaner, Carson City, NV) copper grid. The protein was allowed to adsorb for 5 min. After wicking away the excess solution with a blotting paper, the grid was negatively stained four times, each time using 4 μL of 2 % aqueous uranyl acetate for 1 min. Between each staining step, the excess of uranyl acetate was wicked away with a blotting paper. Finally, the grid was air dried for 30 min before imaging. TEM images were taken with the HITACHI HT7700 transmission electron microscope (Hitachi High Technologies Corp., Tokyo, Japan). The beam current and the accelerating voltage were 8.0 μA and 80 keV, respectively. Images were analyzed by using ImageJ. The fibril widths and lengths were estimated from 5 different images.

### 4.5.7 Circular dichroism spectroscopy

The secondary structures of unincubated Aβ40 monomers and fibrils produced after 21 days of incubation were analyzed by circular dichroism spectroscopy (CD). The protein solution was first desalted by transferring 250 μL of Aβ at 100 μM into an Amicon Centrifugal Filter (Millipore Sigma, Burlington, MA) with 3 KDa cutoff. 4 mL of 10 mM PB was added and the protein was centrifuged at 3,500 rpm for 10 min. This washing step was done twice and the final protein-containing retentate was brought back to 250 μL. The protein concentration was checked by Bradford assay (Sigma-Aldrich, St. Louis, MO) and the protein was diluted to 25 μM (108 μg/mL) in 10 mM PB and analyzed on AVIV 410 CD Spectrometer (AVIV, Lakewood, NJ) in a quartz spectrophotometer cell with a path length of 1 mm (Starna cells Inc, Atascadero, CA). CD spectra were collected between 190 - 300 nm with an average reading time of 15 s. Data were then converted into molar ellipticity [θ].
4.5.8 Binding constants

The binding constants of ThT, OPE1- and OPE2+ to fibrillar Aβ40 was obtained from a constant protein concentration binding assay \(^\text{211}\). Triplet samples of 5 µM protein and varying concentrations of sensor (0 to 30 µM for ThT, or 0 to 2 µM for OPE1- and OPE2+) were prepared and emission spectra were obtained for each sample. Background (sensors alone) subtracted integrated intensities were calculated and plotted against sensor concentration for each sensor and the binding constants were fitted using a nonlinear regression curve assuming site specific binding and using a Hill coefficient to account for any binding cooperativity.

4.5.9 Size exclusion high performance liquid chromatography (SEC)

The unincubated and incubated Aβ40 and Aβ42 samples were analyzed by SEC to separate and quantify the amounts of soluble oligomers and monomers present in the samples. Before injecting the sample on the HPLC column, 70 µL of 100 µM (Aβ40) or 45 µM (Aβ42) was centrifuged for 15 min at 14,000 rpm. Supernatant (65 µL) was injected on a BioSec-SEC-s3000 (Phenomenex, Torrance, CA) column that was already equilibrated with 10 mM phosphate buffer saline (PBS) at pH 7.4 at 0.5 mL/min on Agilent 1100 series system (Agilent Technology, Santa Clara, CA). Absorbance at 215 nm was monitored. Background signal was subtracted using Agilent ChemStation software and percentage of soluble protein was calculated relative to the proteins present in the unincubated samples.

4.5.10 Dynamic light scattering (DLS)

The hydrodynamic radii (R\(H\)) of Aβ42 samples were determined by DLS. Samples at 45 µM were centrifuged (15 min at 14,000 rpm) to remove large particles and dust. Supernatant was loaded into a quartz microcuvette and analyzed with a DAWN HELEOS II light scattering detector (Wyatt Technology Corporation, Santa Barbara, CA). Scattering intensity was
collected at 90˚ of the incident beam and recorded for 10 min. Data were analyzed using the cumulant analysis method using the ASTRA 5.3.4.20 software to yield $R_H$ values $^{212}$.

4.5.11 **Statistical analysis**

Statistical analysis was carried out using JMP Pro 13 (Cary, NC). The integrated intensities of the emission spectra obtained from the sensor alone, the sensor in the presence of monomers or fibrils were compared by using a two-tailed t-test with a p-value ≤ 0.05.

### 4.6 Author Information

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4.6.2 **Author Contributions**


4.6.3 **Funding Sources**

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4.7 Acknowledgment

We are thankful to Dr. Yanli Tang and Dr. Eunkyung Ji who originally synthesized the OPE compounds. We thank Dr. Park (Chosun University, South Korea) for providing the Aβ42 plasmid, as well as Dr. Roland Riek (ETH Zurich) for providing the plasmids coding for the four isoforms of α-synuclein.

4.8 Supplementary Information

Figure S1: Size exclusion chromatograms of Aβ40 monomers (unincubated) and fibrils (incubated for 21 days) in Tris buffer (A) or PB buffer (B). 65 µL at 100 µM samples were centrifuged and analyzed by HPLC SEC.
**Figure S2:** Circular dichroism spectra of unincubated Aβ40 in two different solutions.
Figure S3: Absorbance spectra of ThT (A1 and B1), OPE1- (A2 and B2) and OPE2+ (A3 and B3) in the presence of monomeric (blue) and fibrillar (red) Aβ40 incubated in pH 8.0 Tris (A) or pH 7.4 PB (B), compared to sensor alone (black). The sensor concentration was 1 µM and the protein concentration was 5 µM.
**Figure S4**: Integrated intensity of ThT, OPE1-or OPE2+ (1 µM) in the presence of monomeric and fibrillar Aβ40 (5 µM) incubated in pH 8.0 Tris (A) and pH 7.4 PB (B). A t-test was run to evaluate the significance of the difference between sensor alone, un-incubated (Day 0) and 21-day incubated (Day 21) Aβ40. The asterisks indicate a significance difference between two conditions compared with a p-value ≤ 0.05.

**Figure S5**: Linear correlations between fluorescence integrated area of ThT (A), OPE1- (B) and OPE2+ (C) (1 µM) versus Aβ40 concentration. Data were fitted to a linear fit on OriginPro with $R^2 \geq 0.95$. The intercept and slopes values are summarized in the table below.
**Figure S6:** Binding assay profiles of ThT (A1 and B1), OPE1- (A2 and B2) and OPE2+ (A3 and B3) to Aβ40 fibrils produced in pH 8.0 Tris (A) or pH 7.0 PB (B). These saturation binding assay curves were obtained by fitting the data to a nonlinear regression assuming site specific binding with binding cooperativity on OriginPro 9. Experiments were performed in duplicates and error bars represent standard deviations. The maximum specific binding (Bmax), the Hill coefficient (h), the dissociation constant (Kd) and the R² values are summarized in the table above. OPE has been shown to form complexes at concentration higher than 10 µM which leads to fluorescence red-shift and peak sharpening. To isolate OPE/fibrils binding from OPE complexation the binding assay was done at OPE concentration lower than 2 µM.

<table>
<thead>
<tr>
<th>Graph number</th>
<th>Sensor</th>
<th>Protein</th>
<th>Bmax (10^5)</th>
<th>h</th>
<th>Kd (µM)</th>
<th>R²</th>
</tr>
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<tr>
<td>A1</td>
<td>ThT</td>
<td>Aβ40 TRIS</td>
<td>2.23 ± 0.35</td>
<td>1.02 ± 0.31</td>
<td>2.73 ± 1.01</td>
<td>0.95</td>
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<tr>
<td>B1</td>
<td></td>
<td>Aβ40 PB</td>
<td>3.43 ± 0.49</td>
<td>0.87 ± 0.19</td>
<td>5.12 ± 1.98</td>
<td>0.96</td>
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<tr>
<td>A2</td>
<td>OPE1-</td>
<td>Aβ40 TRIS</td>
<td>26.65 ± 2.85</td>
<td>1.26 ± 0.13</td>
<td>0.70 ± 0.14</td>
<td>0.99</td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td>Aβ40 PB</td>
<td>18.19 ± 1.11</td>
<td>1.54 ± 0.23</td>
<td>0.27 ± 0.04</td>
<td>0.96</td>
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<tr>
<td>A3</td>
<td>OPE2+</td>
<td>Aβ40 TRIS</td>
<td>78.23 ± 3.70</td>
<td>1.56 ± 0.26</td>
<td>0.16 ± 0.02</td>
<td>0.97</td>
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<tr>
<td>B3</td>
<td></td>
<td>Aβ40 PB</td>
<td>201.9 ± 8.27</td>
<td>1.68 ± 0.2</td>
<td>0.25 ± 0.02</td>
<td>0.98</td>
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Figure S7: Size exclusion chromatograms of Aβ42 unincubated, 2.4 hr incubated and 24 hr incubated at room temperature. 65 µL of 45 µM samples were centrifuged (15 min at 14,000 rpm) to remove insoluble species and the supernatant was injected into the size exclusion column (BioSep-SEC-S3000 from Phenomenex).

Figure S8: TEM images of α-synuclein WT (A) and A30P (B) after 6 days of incubation.
Chapter 5: Characterization of Oligomeric p-Phenylene Ethynylene as an Amyloid Probe: Background Fluorescence, Effect on Aggregation, and Cell Toxicity

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5.1 Abstract

Amyloid aggregates are believed to play a key role in the development of neurodegenerative disorders. Currently, amyloid aggregation is mainly investigated by using fluorescent amyloid probes such as Thioflavin T (ThT). These probes lack sensitivity for the detection of early aggregates (oligomers and protofibrils) causing a poor characterization of the early event of protein aggregation. Oligomeric p-phenylene ethynlenes (OPE) are a class of fluorescent probe previously described to detect selectively both fibrillar and pre-fibrillar conformations of amyloid proteins over their monomeric counterpart. The detection of early pre-fibrillar aggregates makes OPE highly promising to shine light on amyloid aggregation kinetic and pathways. In this study, we further characterized the negatively charged OPE1− as an amyloid probe. We found that OPE optimal concentration for amyloid aggregates sensing was below 8 µM at which OPE1− background fluorescence was minimal. At concentration higher than 10 µM, OPE1− formed super-luminescent complexes which might interfere with its sensing properties. We also evaluated the effect of OPE1− on Aβ fibrillation during co-incubation at
molar ratio OPE to protein 1:2, 1:5 and 1:10. During co-incubation OPE\textsuperscript{1,2} did not alter neither the fibrillation kinetic nor the aggregates morphology, hydrophobicity or neurotoxicity. These results indicate that OPE\textsuperscript{1,2} could be used in a co-incubation system to track protein aggregation through real-time fluorescence imaging which could lead to a better understanding of amyloid aggregation kinetic essential for the development of novel therapeutic strategy.

**Keywords:** neurodegenerative diseases, protein aggregate detection, fluorescent optical probes, oligomeric \(p\)-phenylene ethynylenes, amyloid aggregation, neurotoxicity

### 5.2 Introduction

Abnormal protein misfolding and aggregation into \(\beta\)-sheet rich aggregates or amyloids, is a common pathological hallmark of neurodegenerative disorders (ND) such as Alzheimer’s (AD) and Parkinson’s (PD) diseases\textsuperscript{61, 213}. A large array of proteins have been found to form amyloid aggregates in ND including amyloid-\(\beta\) (A\(\beta\)), tau and \(\alpha\)-synuclein proteins.\textsuperscript{213} Amyloid aggregates are believed to be produced through a nucleation-dependent fibrillation mechanism where the misfolded protein forms small oligomers and protofibrils which act as seed for the growth of long fibrillar aggregates rich in \(\beta\)-sheet\textsuperscript{6-8, 10, 11}. This aggregation mechanism is complex and is still poorly understood.

The current understanding of amyloid aggregation is mainly based on the use of amyloid probes such as Thioflavin T (ThT). This histological probe detects amyloid aggregates through hydrophobic interaction which results into fluorescence turn-on and spectral shift\textsuperscript{47}. Recently ThT’s fluorescent turn-on property was also used to image protein fibrillation in real-time through fluorescence-based microscopy methods such as internal reflection fluorescence
microscopy (TIRFM),\textsuperscript{214} or polarized super-resolution structural imaging\textsuperscript{215,216}. By using ThT in real-time imaging, amyloid fibrillation rate was extracted and the heterogeneity of the amyloid structures was revealed\textsuperscript{214}. Despite the efficiency of ThT as an amyloid protein,\textsuperscript{217} ThT lacks of sensitivity for the detection of early aggregates (oligomers and protofibrils)\textsuperscript{218,219} which limits its use to monitor early aggregation events essential to shine light on the aggregation dynamic.

Oligomeric $p$-phenylene ethynlenes (OPEs) are novel fluorescence sensors previously described to selectively detect fibrillar and pre-fibrillar aggregates made of two disease-relevant proteins, Aβ and α-synuclein proteins\textsuperscript{220}. The negatively charged OPE\textsuperscript{1,2} characterized by one repeat unit and two side chains both terminated with a sulfonate group (Figure 5-1), was characterized by higher selectivity and affinity for the detection of Aβ fibrils than ThT. Also, OPE\textsuperscript{1,2} was shown to interact with pre-fibrillar aggregates that ThT failed to detect\textsuperscript{220}. This selective sensing is based on OPE\textsuperscript{1,2} interaction with the hydrophobic β-sheet structure of amyloid aggregates causing OPE’s backbone planarization, hydrophobic unquenching and formation of super-luminescent complexes\textsuperscript{81,82,84}. OPE’s high fluorescence enhancement and its capability to detect early aggregates makes it highly promising to better understand amyloid aggregation kinetic through real-time imaging.

In this study, we propose to further characterize OPE\textsuperscript{1,2} as an amyloid sensor. This characterization consisted in first evaluating OPE\textsuperscript{1,2} background fluorescence to determine optimal OPE\textsuperscript{1,2} concentration for amyloid sensing. Secondly, the effect of OPE on amyloid aggregation when co-incubated with Aβ40 was analyzed. Finally, the effect of co-incubation on fibrils morphology, hydrophobicity and toxicity were investigated. Thanks to this study, we
gained a better understanding of OPE as an amyloid sensor which opens the door to their use in real-time fluorescence microscopy for amyloid aggregation monitoring.

![OPE1^2- structure](image)

**Figure 5-1: OPE1^2- structure**

### 5.3 Results and Discussion

OPE was previously described to detect selectively fibrillar and pre-fibrillar aggregates made of Aβ40 and α-synuclein proteins over the monomeric counterpart through fluorescence turn-on\(^{220}\). This selective binding makes OPE promising to track amyloid aggregation. This study consisted in further characterizing the negatively charged OPE1^2- as an amyloid probe.

#### 5.3.1 OPE1^2- forms super-luminescent aggregates above 10 µM

The efficacy of a fluorescence-based amyloid probe such as OPE1^2- depends on its low background fluorescence when free in solution. To determine the optimal OPE concentration for amyloid sensing, we investigated its background fluorescence in phosphate buffer (PB) between 0 to 15 µM (**Figure 5-2**).
Figure 5-2: OPE$_{1}^2^-$ background fluorescence in phosphate buffer. OPE$_{1}^2^-$ emission spectra between 0 to 15 µM (A) was recorded. (B) and (C) are zoom-in of the emission spectra obtained at concentration between 0 and 10 µM (B) or between 0 and 1 µM (C). (D) Integrated emission areas were plotted in function of OPE concentration.

At concentration below 0.2 µM (Figure 5-2C), OPE$_{1}^2^-$ background fluorescence was similar to phosphate buffer (PB) fluorescence indicating that OPE$_{1}^2^-$ was fully quenched by water molecules. This efficient quenching is ensured by a rapid deactivation of OPE$_{1}^2^-$ excited singlet state through proton transfer with the surrounding water molecules$^{83, 84, 221}$. At 0.5 µM OPE$_{1}^2^-$ background emission intensity increased by 1 fold and gave rise to a broad emission peak centered at around 470 nm (Figure 5-2 C) which indicates the formation of disordered micelle-like structure likely made of H-type aggregates$^{192}$. The intensity of this emission peak linearly increased and red shifted (~ 495 nm) with increase in OPE concentration up to 10 µM.
At 10 µM a new shoulder appeared on the emission spectrum at around 445 nm and became sharper at 15 µM where the fluorescence intensity increased by more than 100-fold compared to PB background (Figure 5-2A and B). This large fluorescence enhancement accompanied by an emission blue shift and peak sharpening indicate the formation of OPE$_{1}^{2-}$ super-luminescent complexes mainly made of J-aggregates.$^{85}$

The formation of super luminescent complexes at concentration above 10 µM might interfere with OPE$_{1}^{2-}$ sensing properties. These data allowed us to set up an optimal OPE$_{1}^{2-}$ concentration range for amyloid sensing between 0.1 – 8 µM, which supports previous studies where OPE$_{1}^{2-}$ was commonly used at 1 µM.$^{81, 82, 220}$

5.3.2 OPE$_{1}^{2-}$ does not alter Aβ40 fibrillation kinetic and aggregates morphology

Amyloid aggregation is commonly monitored by using amyloid fluorescent probe in a co-incubation system where both the probe (ThT or Congo Red) and amyloid protein are incubated together during aggregation.$^{222, 223}$ The risk of co-incubation is that the probe can interfere with the aggregation. In this study, we are evaluating the effect of OPE$_{1}^{2-}$ on fibrillation kinetic in a co-incubation system. We compared Aβ40 aggregation in the absence and presence of OPE$_{1}^{2-}$ at three different molar ratio Aβ to OPE$_{1}^{2-}$: 2 to 1, 5 to 1, and 10 to 1 (Table 5-1). Fibrillation of Aβ40 incubated alone was monitored by using the well-known amyloid probe ThT at 20 µM in the presence of 5 µM Aβ as well as with 1 µM OPE in the presence of 2, 5 or 10 µM Aβ. For the co-incubated conditions, Aβ aggregation was only monitored through OPE fluorescence after diluting the sample to reach 1 µM OPE which led to Aβ concentration of 2, 5 or 10 µM depending on the co-incubation condition. The integrated emission areas of ThT and OPE were calculated after background subtracted and are reported
**Figure 5-3A1** and **B1**. Integrated emission areas were also normalized with the average value measured during the last two days of incubation as the aggregation kinetic reached plateau at this stage (**Figure 5-3A2** and **B2**). The large standard deviations of the fluorescence data prevented any fitting to extract the aggregation growth parameters such as lag phase or growth rate.

**Table 5-1:** Aβ and OPE$_{1^2}$ concentrations used for the four tested conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[Aβ] μM</th>
<th>[OPE] μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ alone</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>Aβ:OPE 2:1</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Aβ:OPE 5:1</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>Aβ:OPE 10:1</td>
<td>80</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 5-3: A: Integrated fluorescence intensity profiles (A1) and normalized fluorescence profiles (A2) of ThT or OPE$_{1}^{2-}$ in the presence of Aβ40 (80 µM) incubated in 50 mM PB pH 7.4 buffer containing 100 mM NaCl at different time points. Fluorescence was recorded at 20 µM ThT and 5 µM Aβ or 1 µM OPE$_{1}^{2-}$ and varying concentration of Aβ40 (2, 5 or 10 µM). B: Integrated fluorescence intensity profiles (B1) and normalized fluorescence profiles (B2) of Aβ (80 µM) incubated alone (black solid line) or co-incubated with varying concentration of OPE$_{1}^{2-}$ (40, 16 and 8 µM) in 50 mM PB and 100 mM NaCl at 37 °C at different time points. Fluorescence of Aβ incubated alone was collected in the presence of 20 µM ThT and 5 µM Aβ (black solid line). Fluorescence of co-incubated samples diluted to 1 µM OPE$_{1}^{2-}$ was also collected at varying Aβ concentration (dashed lines).

To compare ThT and OPE$_{1}^{2-}$ sensing properties, the aggregation of Aβ40 incubated alone was monitored by using both sensors (Figure 5-3A1 and A2). ThT fluorescence was recorded at 20 µM which is commonly used for this probe to ensure maximal fluorescence enhancement,$^{217}$ while OPE$_{1}^{2-}$ was used at 1µM. The aggregation profile generated with ThT
showed an exponential aggregation without a lag phase which reached plateau after around 6 days (Figure 5-3A2). When OPE was used at 1 µM to track the aggregation of the same protein, a similar aggregation profile was observed. OPE was previously described to detect early aggregates that ThT failed to detect (α-synuclein oligomers and Aβ42 oligomers)\(^{220}\). Similar aggregation profiles obtained by both ThT and OPE might indicate a low percentage of oligomers that OPE was not able to detect. Additionally, as shown Figure 5-3A1, ThT emission intensity recorded in the presence of 20 µM ThT and 5 µM protein (black line) was two-fold lower than OPE fluorescence recorded in the presence of the same protein concentration but with 20-fold less OPE concentration (1 µM OPE 5 µM Aβ - red line). OPE stronger fluorescence signal makes this probe superior than ThT to track amyloid fibrillation.

The effect of OPE\(_{1^{-2}}\) on Aβ40 aggregation was then investigated by monitoring OPE\(_{1^{-2}}\) fluorescence in co-incubated samples and by comparing this kinetic to the one obtained from Aβ incubated alone through ThT fluorescence (Figure 5-3B1 and B2). As previously observed, OPE fluorescence intensity was higher than ThT (Figure 5-3B1). Despite this difference in emission intensity, fluorescence profiles obtained from all three co-incubated conditions (2:1, 5:1 and 10:1 molar concentration Aβ to OPE\(_{1^{-2}}\)) showed a similar aggregation kinetic compared to Aβ incubated alone characterized by a rapid exponential growth of fibrils that reached plateau at around day 6 (Figure 5-3B2). These data indicate that OPE\(_{1^{-2}}\) did not alter Aβ fibrillation. To further characterize the effect of OPE on aggregation kinetic, more particularly on the formation of oligomers, Aβ40 should be analyzed during aggregation by size exclusion (SE) HPLC or by western-blot using the appropriate antibodies (anti-Aβ 6E10, anti-amyloid fibrils OC and anti-amyloid oligomers A11)\(^{65, 70}\).
To further evaluate the effect of OPE$_{1}^{2-}$ on Aβ aggregates morphology, TEM images were taken during the incubation. As shown Figure 5-4, when Aβ40 was incubated alone, small oligomers were produced after 2 days of incubation. At day 5 and 9 longer protofibrils were present and after 14 days, mature fibrils were observed. Interestingly, no difference between day 9 and day 14 was observed through ThT and OPE fluorescence which might indicate that the amount of protofibrils present at day 9 might be similar to the quantity of mature fibrils present at days 15. When Aβ40 was co-incubated with OPE$_{1}^{2-}$ (2:1, 5:1 and 10:1 molar ratio Aβ to OPE), similar aggregates size and morphology were observed compared to Aβ incubated alone, indicating that OPE$_{1}^{2-}$ did not alter the aggregation kinetic nor the aggregation pathway. These results show that OPE$_{1}^{2-}$ can be used in a co-incubation experiment to track amyloid fibrillation without altering amyloid aggregation.
Figure 5-4: TEM images of Aβ alone and Aβ co-incubated with OPE$_{1}^{2-}$ at 2:1, 5:1 and 10:1 molar concentration over 14 days of incubation at 37 °C.

5.3.3 OPE$_{1}^{2-}$ displays a different binding mode when co-incubated with Aβ40 during fibrillation

When co-incubated with Aβ40 peptide, OPE$_{1}^{2-}$ is an efficient probe to track fibrillation as it does not alter protein aggregation and maintains its selective fluorescence turn-on properties. We previously observed that OPE$_{1}^{2-}$ forms super-luminescent complexes at concentration higher than 10 μM, which leads to high background fluorescence and red shift in the emission spectrum. During co-incubation with Aβ40 at 80 μM, OPE$_{1}^{2-}$ concentration was between 8 to 40 μM. At these concentrations OPE$_{1}^{2-}$ might have formed super-luminescent complexes
which might have influenced OPE$_{1}$ binding to the amyloid aggregates. To further understand OPE$_{1}$ binding mode, we reported OPE$_{1}$ emission spectra obtained from Aβ40 incubated alone and from co-incubated samples (Figure 5-5). OPE fluorescence was monitored at 1 µM in the presence of 2, 5 or 10 µM peptide.

**Figure 5-5:** OPE$_{1}$ emission spectra (1 µM) generated in the presence of pre-formed aggregates (Aβ40 incubated alone) (A, B and C) or from co-incubation (D, E and F). Three OPE to peptide ratios were used for fluorescence monitoring 2:1, 5:1 and 10:1. OPE and Aβ concentrations used for fluorescence analysis are reported on the left inside. The concentration of Aβ and OPE used during incubation are reported on the top left of each plot.
At 1 µM OPE\textsubscript{1} was highly quenched in phosphate buffer with a weak emission peak centered at 471 nm (Figure 5-5). OPE\textsubscript{1} fluorescence significantly increased in the presence of Aβ40 aggregates generated after 1, 5, 9 and 14 days of incubation (Aβ40 alone) (Figure 5-5 A, B and C). Overall, in the presence of pre-formed Aβ40 aggregates OPE\textsubscript{1} emission peak was sharper and was blue shifted at around 450 nm (between 444 - 453 nm depending on the incubation days and protein concentration), which indicates the formation of OPE J-dimers, as previously described\textsuperscript{220}.

Aβ samples obtained from co-incubation with OPE\textsubscript{1} were also analyzed after dilution in phosphate buffer to reach 1 µM OPE. In this condition, OPE\textsubscript{1} fluorescence had significantly higher intensity compared to OPE\textsubscript{1} fluorescence generated in the presence of pre-formed Aβ40 aggregates (1.5 to 6-fold higher). The conditions 1:2 and 1:5 OPE\textsubscript{1} to peptide were characterized by the appearance of a new spectral signature in addition to the increase in fluorescence intensity (Figure 5-5 D and E). This new emission spectrum was characterized by the presence of a sharp peak centered at around 450 nm indicating the presence of rigid J-aggregates and a shoulder located at 476 nm representative of disordered H-aggregates. OPE was previously described to form J-aggregates when present at concentration above 10 µM (Figure 5-2). Based on this information and the observation of a sharper peak at 450 nm after co-incubation, we hypothesized that OPE interacted with the fibrils as a complex made of J-aggregates which did not breakdown after dilution in PB. This observation indicates that OPE can sense Aβ40 aggregates as a single molecule or as a complex, similarly to what was reported for other small molecules such as curcumin\textsuperscript{224}. Despite a difference in the binding mode, OPE\textsubscript{1} maintained its sensing propriety to track amyloid aggregation during co-incubation.
5.3.4 \textit{OPE}_1^2 \textit{does not alter fibrils hydrophobicity}

The effect of \textit{OPE}_1^2 on Aβ fibrils hydrophobicity was also investigated with the fluorescent probe Nile Red (NR). NR was previously described to interact selectively to hydrophobic surfaces leading to fluorescence turn-on as well as emission blue shift\textsuperscript{225}. Additionally, NR fluorescence does not overlap with \textit{OPE}_1^2 and no fluorescence resonance energy transfer (FRET) are expected when measuring NR fluorescence (NR excitation: 550 nm, NR emission: 620 nm) in the presence of \textit{OPE}_1^2 (\textit{OPE}_1^2 excitation: 380 nm, \textit{OPE}_1^2 emission: 450 nm). NR fluorescence was monitored at 1 µM in the presence of 10 µM Aβ40 monomers or 10 µM Aβ40 fibrils produced in the absence and presence of \textit{OPE} (Figure 5-6A and B).

![Figure 5-6: Nile red emission spectra in the presence of Aβ monomers and Aβ fibrils generated from Aβ incubated alone and OPE:Aβ co-incubated at 1:2, 1:5 and 1:10. Aβ monomers and fibrils were washed with PB by using an Amicon falcon to remove any excess of free \textit{OPE}_1^2, DMSO and sodium azide. NR emission was monitored at 1 µM in the presence of 10 µM Aβ peptide at the excitation wavelength 550 nm. A. NR emission spectra. B. Bar plot of NR integrated emission intensity from triplicate samples.](image)

NR was fully quenched in phosphate buffer with a weak emission peak centered at around 650 nm (Figure 5-6A). In the presence of Aβ40 monomers, NR fluorescence stayed
unchanged. NR fluorescence drastically increased in the presence of Aβ40 fibrils to reach around 2,000 counts/sec which was accompanied by an emission blue shift at around 611 nm (Figure 5-6A). This change in NR fluorescence signature indicates that Aβ40 fibrils are more hydrophobic than Aβ40 monomers.

The influence of OPE$_{1}^{2-}$ on NR binding to Aβ fibrils was evaluated by recording NR fluorescence in the presence of pre-formed Aβ fibrils to which OPE$_{1}^{2-}$ was added at a final concentration of 5 µM. NR fluorescence generated in the presence of OPE was similar to the fluorescence spectra obtained in the presence of Aβ fibrils alone, indicating that OPE$_{1}^{2-}$ interaction with Aβ fibrils did not induce any changes in NR binding. NR fluorescence was also monitored in the presence of Aβ40 fibrils produced from co-incubation with OPE$_{1}^{2-}$. No significant difference was observed in terms of NR fluorescence intensity (Figure 5-6B) or emission shift (Figure 5-6A) by comparison with pre-formed fibrils.

Overall, we observed that Aβ40 fibrils were intrinsically more hydrophobic than the monomeric species. More importantly, OPE$_{1}^{2-}$ did not alter fibrils hydrophobicity when co-incubated with the peptide.

5.3.5 OPE$_{1}^{2-}$ does not modify Aβ cytotoxicity

When co-incubated with Aβ, OPE$_{1}^{2-}$ did not alter neither the aggregation kinetic nor the overall hydrophobicity. In order to investigate the effect of OPE$_{1}^{2-}$ on fibrils neurotoxicity we evaluated both OPE$_{1}^{2-}$ and fibrils toxicity on SHSY-5Y neuroblastoma cells (Figure 5-7A and B).
Figure 5-7: SHSY-5Y cell viability after treatment with OPE$_{1}^{2-}$ and Aβ40 fibrils. A. OPE$_{1}^{2-}$ cell toxicity was investigated at concentration between 1 to 10 µM after 24 hours treatment. B. Aβ fibrils toxicity was evaluated after 48 hours at 20 µM. Before treatment, Aβ fibrils were washed with 10 mM PB at pH7.4. Cell viability was normalized to the negative control, untreated cells. Error bars represent standard deviations between quintuplet experiments. The red line represents 70% cell viability, which is used as a threshold for cytotoxicity.

As shown Figure 5-7A, cell treated with OPE$_{1}^{2-}$ at concentration between 1 to 10 µM displayed similar viabilities compared to the untreated cells indicating that OPE$_{1}^{2-}$ is not cytotoxic. Aβ fibrils generated from the four incubations previously described were also tested. These fibrils were first washed with 10 mM PB pH 7.4 to remove any free OPE$_{1}^{2-}$, DMSO, and sodium azide. Protein concentration was then determined by Bradford assay and neuroblastoma cells were treated with 20 µM fibrils. As shown Figure 5-7B, cell viability was not affected by the presence of Aβ fibrils produced from Aβ incubated alone. Aβ fibrils low neurotoxicity corroborates previous studies showing that the most toxic species are Aβ oligomers$^{226-228}$. When neuroblastoma cells were treated with Aβ fibrils produced in the presence of OPE$_{1}^{2-}$ at molar ratio 2:1, 5:1 and 10:1, no change in cell toxicity was observed indicating that the binding of OPE$_{1}^{2-}$ to Aβ fibrils during aggregation, did not modify their toxicity.
These results indicate that OPE$_1^{2-}$ is not cytotoxic and it does not change fibrils overall toxicity which make this probe applicable to track Aβ fibrillation in a cell culture environment.

5.4 Conclusion

This study consisted in characterizing OPE as an amyloid probe in terms of background fluorescence, effect on amyloid aggregation and cell toxicity. We showed that OPE$_1^{2-}$ background fluorescence was minimal at concentration below 8 µM which supports the use of this probe at 1 µM as previously reported$^{81,82,220}$. Besides, OPE$_1^{2-}$ formed J-aggregates at concentration higher than 10 µM leading to high fluorescence turn-on which might interfere with its sensing properties. Additionally, we evaluated OPE$_1^{2-}$ sensing of Aβ40 fibrillation during co-incubation with the peptide. We found that OPE$_1^{2-}$ did not alter the aggregation kinetic even when co-incubated at the high molar ratio 1:2 OPE$_1^{2-}$ to protein and maintained its sensing properties, indicating that OPE$_1^{2-}$ could be used to monitor protein aggregation through real-time fluorescence microscopy. Finally, we showed that OPE$_1^{2-}$ did not display any cell toxicity and more importantly did not affect fibrils neurotoxicity. The fact that OPE$_1^{2-}$ can detect early aggregates and does not alter neither amyloid aggregation nor aggregates toxicity makes this probe highly promising to shine light on amyloid aggregation kinetic implicated in the development of neurodegenerative disorders.

5.5 Experimental Methods

5.5.1 Material

Synthetic amyloid β-40 (Aβ40) was purchased form Peptide2.0 (Chantilly, VA). Dimethyl sulfoxide (DMSO), sodium chloride (NaCl) and sodium azide were acquired from EMD
Millipore (Burlington, MA). Sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrous used to prepare phosphate buffer were purchased from Thermo Fisher Scientific (Waltham, MA). 400 mesh copper grids covered by 5-10 nm formvar/carbon film were purchased from Ted Pella (Redding, CA). 2% aqueous uranyl acetate was purchased from Electron Microscopy Sciences (Hatfield, PA). Thioflavin T (ThT) and Nile red (NR) were acquired from ACROS Organics (Belgium) and Sigma-Aldrich (St. Louis, MO), respectively.

$\text{OPE}_{12}^{2-}$ was synthesized and purified by previously published procedures (MW: 724.79 g/mol; $\varepsilon_{370 \text{ nm in water}} = (3.92 \pm 0.013) \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$)\textsuperscript{181}. SH-SY5Y neuroblastoma cells, Dulbecco’s Modified Eagle’s Medium (DMEM) F12 media, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin (PS) at 10,000 U/mL was purchased from Thermo Fisher Scientific (Waltham, MA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was acquired from Promega (Madison, WI).

5.5.2 $\text{A}\beta40$ preparation and incubation

The lyophilized A$\beta$40 peptide was solubilized in DMSO at 50 mg/mL and was centrifuged for 10 min at 14,000 rpm. The soluble peptide was transferred in a new vial and the protein concentration was determined by measuring the absorbance at 280 nm with the Nanodrop (Thermo Fisher Scientific, Waltham, MA) and using A$\beta$40 coefficient of extinction and molecular weight ($\varepsilon_{280\text{nm}} = 1,490 \text{ M}^{-1}\text{cm}^{-1}$; MW: 4,329 g/mol). A$\beta$40 was then diluted to reach 80 $\mu$M in 50 mM PB pH 7.4 buffer containing 100 mM NaCl and 0.01 % w/v sodium azide and was incubated at 37 °C under quiescent condition for up to 15 days.

5.5.3 Fluorescence measurement

Fibrils formation was monitored by using Thioflavin T and OPE$_{12}^{2-}$ fluorescence. Fluorescence spectra were recorded with the PTI QuantaMaster 40 steady state spectrofluorometer.
ThT and OPE\textsubscript{1\textsuperscript{2}} were mixed with Aβ40 peptide at the following concentration: 20 µM ThT with 5 µM Aβ and 1 µM OPE with 2, 5 or 10 µM Aβ. After incubating the sample for 30 min at room temperature, the emission spectra were recorded by using the excitation wavelengths of 440 and 390 nm for ThT and OPE\textsubscript{1\textsuperscript{2}}, respectively.

Nile red (NR) was used to monitor fibrils hydrophobicity by mixing NR with Aβ40 sample at 1 to 10 molar ratio (1 µM NR with 10 µM Aβ40). After incubating the sample for 30 min at room temperature, NR emission spectrum was recorded at 550 nm.

5.5.4 TEM imaging

Aβ40 was imaged during incubation by Transmission Electron Microscopy (TEM). The peptide diluted at 5 µM in MilliQ water was loaded onto a carbon grid exposed to glow discharge for 30 sec. The peptide was let to adsorb for 5 min after which the excess of solution was wick away. The peptide was then stained by using uranyl acetate at 2% through four consecutive staining steps: the first one consisted in staining the sample with 4 µL of uranyl acetate for 3 min, the last three staining were done for 1 min. After wicking away the excess of stain the grid was air dried for 30 min before imaging with the HITACHI HT7700 transmission electron microscope (Hitachi High Technologies Corp., Tokyo, Japan). The beam current and the accelerating voltage used during imaging were 8.0 µA and 80 keV, respectively.

5.5.5 Cell toxicity

SHSY-5Y cells were used to evaluate OPE\textsubscript{1\textsuperscript{2}} cell toxicity. The neuroblastoma cells were cultivated in a T-75 flask (Thermo Fisher Scientific, Waltham, MA) up to 80% confluency. After detaching the cells with 0.25% trypsin solution, media was added to the flask and the floating cells were centrifuged at 2,000 rpm for 5 min. Cells were resuspending in a fresh
DMEM media containing 1% PS and 10% FBS, and cell density was measured by using a hemocytometer. Cells were diluted to 200,000 cells/mL, were loaded in a 96 well plate (100 µL/well) and were then incubated overnight at 37 °C and 5% CO₂. Media was changed with DMEM media containing 1%PS but depraved in FBS to ensure cell synchronization to a quiescent stage G0. Cells were incubated overnight after which fresh media containing OPE₁²⁻ at different concentrations were added to the cells. Cell viability was monitored after 24 hours of incubation with MTS assay. Briefly, 20 µL of MTS reagent was added to the 100 µL media present in each well and the 96 well plate was further incubated at 37°C for another 3 hours. Absorbance at 490 nm was monitored. MTS background absorbance was subtracted, and cell alone were used as 100%.

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5.7 Acknowledgment

We are thankful to Dr. Yanli Tang and Dr. Eunkyung Ji who originally synthesized the OPE compounds.
Chapter 6: Characterization of a Luminescent Sensor for the Selective Detection of Amyloid Aggregates in Cerebrospinal Fluid

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\textsuperscript{†} AMF and FAM equally contributed to this work

\textsuperscript{1}Center for Biomedical Engineering, \textsuperscript{2}Biomedical Engineering Graduate Program, \textsuperscript{3}Departments of Neurology, \textsuperscript{4}Department of Molecular Genetics and Microbiology, \textsuperscript{5}Department of Neurology, \textsuperscript{6}Department of Chemical and Biological Engineering, University of New Mexico, Albuquerque, New Mexico 87131, United States

6.1 Abstract

Amyloid aggregates deposition in the brain and increase in amyloid-β oligomers concentration in cerebrospinal fluid (CSF) to reach pg/mL range have been correlated with cognitive impairment which make them ideal biomarkers for early Alzheimer’s disease (AD) diagnosis. In this study, we reported the sensing properties of a fluorescent probe, oligomer \( p \)-phenylene ethylene (OPE), for the detection of a large array of amyloid aggregates in phosphate buffer (PB) and in CSF. We also investigated OPE for the detection of pathologic oligomers in CSF isolated from patient diagnosed with dementia through the Aβ-protein misfolding cyclic amplification (Aβ-PMCA) assay. Two oppositely charged OPE were tested, the negatively charged OPE\textsubscript{1}\textsuperscript{2−} and the positively charged OPE\textsubscript{2}\textsuperscript{4+}. In phosphate buffer OPE\textsubscript{1}\textsuperscript{2−} was a superior sensor for the detection of a large array of amyloid aggregates compared to OPE\textsubscript{2}\textsuperscript{4+}. In fact, OPE\textsubscript{1}\textsuperscript{2−} detected fibrils made of insulin, lysozyme, Aβ40 and PHF6 as well as htau oligomers,
while OPE$_2^{4+}$ only detected fibrils composed of insulin, lysozyme and Aβ40. Despite superiority of OPE$_1^{2-}$ in PB, this sensor was more sensitive to its microenvironment as it lost its sensing properties in CSF, while OPE$_2^{4+}$ maintained its selective sensing. Finally, OPE sensing properties were evaluated in CSF isolated from patients with dementia through the Aβ-PMCA assay after spiking the samples with Aβ40 monomers. No detectable fibrillation was observed neither from TEM imaging nor from OPE fluorescence. Further evaluation of the Aβ-PMCA assay would be required to determine the feasibility of this assay to detect picogram range oligomers. Despite OPEs poor performance in CSF isolated from patient with dementia, OPE$_2^{4+}$'s sensing properties in a complex environment could lead to other applications such as the detection of amorphous protein aggregates in protein-based pharmaceuticals.

**Keywords:** oligomer $p$-phenylene ethylene, fluorescent probe, amyloid aggregates detection, cerebrospinal fluids, protein misfolding cyclic amplification

### 6.2 Introduction

Abnormal protein misfolding and aggregation into $\beta$-sheet rich aggregates or amyloids, is one of the main pathological hallmark of Alzheimer’s disease (AD)$^6,213$. In AD, two main proteins have been found to form amyloid aggregates, Amyloid-$\beta$ (Aβ) and tau proteins which accumulate under the form of amyloid plaques and neurofibrillary tangles in AD brains, respectively$^{213, 229, 230}$. These aggregates are produced through a nucleation-based polymerization mechanism in which misfolded proteins aggregate into small oligomers and protofibrils which are then used as seeds to form stable amyloid fibrils$^6-8, 10, 11$. Theses aggregates have been found to accumulate in the brain$^7$ and more recently, Aβ oligomers have
been found to increase in cerebrospinal fluid (CSF) \(^{41,231-233}\) to reach a concentration in the order of pg/mL \(^{234}\). The accumulation of amyloid aggregates in both the brain and CSF of patients suffering of AD was directly correlated with cognitive decline and is believed to start decades before the development of the symptoms, \(^{88,89}\) which make them ideal biomarkers for early disease detection.

To this day, there is a lack of a sensitive method for early diagnosis of AD. Disease diagnosis is mainly based on symptoms evaluation and is confirmed through the detection of amyloid plaques \textit{in vivo} which can be accomplished via Positron Emission Tomography (PET) \(^{50,52,235-237}\). Additionally, other non-PET sensors such as Thioflavin T (ThT) are used for post-mortem tissue analysis and definitive diagnosis \(^{238}\). Current PET and histological probes are of limited application as FDA approved PET probes only detect amyloid plaques made of Aβ protein \(^{54-56,239}\) and current histological probes are not good at detecting the early aggregates, oligomers and protofibrils \(^{56}\). More studies are currently investigating the detection of Aβ oligomers in CSF for AD diagnosis. A promising method which would ensure the detection of Aβ oligomers at concentration as low as pg/mL is based on the Aβ protein misfolding cyclic amplification assay (Aβ-PMCA) \(^{240,241}\). This method consists in using the seeding potency of the oligomers and tracking the formation of amyloid fibrils in CSF with ThT after spiking the sample with monomeric Aβ42 \(^{241}\). To ensure efficient detection of protein aggregates in the brain and in CSF, there is a need to develop new conformation-specific probe highly selective toward amyloid aggregates made of different proteins, with low limit of detection, superior selectivity to amyloid aggregates and low sensitivity to its microenvironment such as the presence of glucose, salt, or non-amyloidogenic protein (albumin).
Oligomeric $p$-phenylene ethynylenes (OPEs) are water-soluble fluorescent probes which were previously described to selectively detect amyloid fibrils rich in β-sheet over their monomeric counterpart\textsuperscript{81, 82, 220}. OPE sensing of amyloid fibrils is ensured through its quenching by water molecules when free in solution and its large fluorescence turn-on upon interaction to the hydrophobic β-sheet structures composing the amyloid aggregates\textsuperscript{83, 178, 181, 182}. This fluorescence enhancement is driven by three main mechanisms: (1) hydrophobic unquenching, (2) backbone planarization and (3) formation of OPE super-luminescent complexes\textsuperscript{81, 82, 87}. The negatively charged OPE\textsubscript{1}\textsuperscript{2−} and the positively charged OPE\textsubscript{2}\textsuperscript{4+} (Table 6-1) have been shown to detect amyloid aggregates made of a large variety of proteins: hen egg-white lysozyme, bovine insulin, Aβ and α-synuclein proteins\textsuperscript{81, 82, 220}. OPE\textsubscript{1}\textsuperscript{2−} presents high binding selectivity toward the fibrillar conformation of all tested proteins,\textsuperscript{81, 82, 220} and was able to detect α-synuclein oligomers but not Aβ42 oligomers\textsuperscript{220}. OPE\textsubscript{2}\textsuperscript{4+} displayed higher fluorescence enhancement compared to OPE\textsubscript{1}\textsuperscript{2−} in the presence of fibrillar aggregates. OPE\textsubscript{2}\textsuperscript{4+} was also better at detecting oligomers made of Aβ42 proteins,\textsuperscript{220} but also presented non-selectively binding to the negatively charged insulin monomers which limited the selective detection of insulin fibrils.\textsuperscript{82}

In this study, we are evaluating the robustness of OPE for the detection of amyloid aggregates in a complex physiological sample, CSF. OPE sensing of amyloid aggregates (fibrils and oligomers) made of a large array of proteins (lysozyme, insulin, tau and Aβ) was quantitatively characterized in terms of selectivity, sensitivity and affinity. OPE background fluorescence and sensing properties were evaluated in CSF. Finally, OPE sensing of amyloid oligomers in CSF isolated from patients with dementia was investigated through the Aβ-PMCA assay.
Table 6-1: OPE$_1^{2-}$ and OPE$_2^{4+}$ structures. OPE$_1^{2-}$ is made of one repeat unit and present two side chains each terminated with a sulfonate group. The positively charged OPE$_2^{4+}$ is composed of two repeat units and four side chains each terminated with a quaternary ammonium group.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPE$_1^{2-}$</td>
<td><img src="image" alt="OPE1.png" /></td>
</tr>
<tr>
<td>OPE$_2^{4+}$</td>
<td><img src="image" alt="OPE2.png" /></td>
</tr>
</tbody>
</table>

6.3 Results and Discussion

6.3.1 Quantitative characterization of OPE sensing of amyloid aggregates

OPE$_1^{2-}$ and OPE$_2^{4+}$ are two fluorescent probes previously described to selectively detect the fibrillar conformation of a variety of amyloid proteins over the monomeric counterpart. To further understand their sensing properties, we evaluated them in the presence of five amyloid aggregates: lysozyme fibrils, insulin fibrils, Aβ40 fibrils, tau PHF6 fibrils and human isolated tau (htau) oligomers (Figure 6-1). Tau PHF6 is the hexapeptide $^{306}$VQIVYK$^{311}$ located in the third repeat unit of the tau microtubule binding domain known to form the main core of tau paired helical filaments (PHF),$^{99,101}$ and was shown to be a relevant tau aggregation model. This large array of amyloid aggregates was used to evaluate the robustness of OPE sensing properties for the detection of highly polymorphic amyloid aggregates characterized by different morphology (Figure 6-1) and net charge (Table 6-2). OPEs sensing was characterized in terms of spectral signature, binding constant and limit of detection.

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Table 6-2: Protein monomers characteristics: molecular weight (MW), isoelectric point (pI) and net charge at pH7.4

<table>
<thead>
<tr>
<th>Monomer parameters</th>
<th>Lysozyme fibrils</th>
<th>Insulin fibrils</th>
<th>Aβ40 fibrils</th>
<th>Tau PHF6 fibrils</th>
<th>hTau oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW (Da)</td>
<td>14,300</td>
<td>5,733</td>
<td>4,329</td>
<td>780</td>
<td>45,849</td>
</tr>
<tr>
<td>Isoelectric point (pI)</td>
<td>9.04</td>
<td>5.59</td>
<td>5.59</td>
<td>8.88</td>
<td>8.24</td>
</tr>
<tr>
<td>Net charge at pH7.4</td>
<td>7.3</td>
<td>-2.4</td>
<td>-2.9</td>
<td>0.8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

OPE$_{1}^{2-}$ and OPE$_{2}^{4+}$ fluorescence was recorded at 1 µM in the presence of the monomeric or aggregated proteins at 21 µg/mL. OPE emission and excitation spectra are presented in Figure 6-2. To quantify the selective detection of the aggregated conformer over the monomeric counterpart, we used the amyloid detection factor (ADF) (Equation 1).

\[
ADF = \frac{F_{(dye+fibrils)} - F_{(dye+monomers)}}{F_{(dye in buffer)}}
\]  

with \(F_{(dye in buffer)}\), \(F_{(dye+fibrils)}\) and \(F_{(dye+monomers)}\) being the integrated emission intensities of the sensor alone, sensor in the presence of fibrils, and sensor in the presence of monomers, respectively. Based on this equation, positive ADF (>1) indicates selective detection of the fibrillar conformers while negative value (<1) indicates higher detection of the monomeric counterpart and ADF value close to 0 (-1<x<1) indicates no selective sensing of either protein conformations. The ADF values generated for both OPE$_{1}^{2-}$ and OPE$_{2}^{4+}$ are summarized in Table 6-3.
Table 6-3: OPE$_{1}^{2-}$ and OPE$_{2}^{4+}$ sensing parameters – amyloid detection factor (ADF), dissociation constant ($k_d$) and limit of detection (LOD)

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Parameters</th>
<th>Lysozyme fibrils</th>
<th>Insulin fibrils</th>
<th>Aβ40 fibrils</th>
<th>Tau PHF6 fibrils</th>
<th>hTau oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPE$_{1}^{2-}$</td>
<td>ADF</td>
<td>4.77 ± 0.53</td>
<td>6.59 ± 0.49</td>
<td>2.36 ± 0.20</td>
<td>8.47 ± 0.63</td>
<td>2.17 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>$k_d$ (µM)</td>
<td>0.59 ± 0.12</td>
<td>0.94 ± 0.13</td>
<td>0.49 ± 0.14</td>
<td>0.84 ± 0.29</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>LOD (µg/mL)</td>
<td>0.85 ± 0.13</td>
<td>0.62 ± 0.05</td>
<td>2.24 ± 0.16</td>
<td>0.52 ± 0.07</td>
<td>4.25 ± 0.41</td>
</tr>
<tr>
<td>OPE$_{2}^{4+}$</td>
<td>ADF</td>
<td>5.96 ± 0.57</td>
<td>5.04 ± 1.32</td>
<td>5.66 ± 0.41</td>
<td>0.72 ± 0.18</td>
<td>-0.28 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>$k_d$ (µM)</td>
<td>0.29 ± 0.06</td>
<td>0.81 ± 0.14</td>
<td>0.16 ± 0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LOD (µg/mL)</td>
<td>0.32 ± 0.06</td>
<td>0.38 ± 0.02</td>
<td>0.28 ± 0.01</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As shown Figure 6-2, at 1 µM both OPE$_{1}^{2-}$ and OPE$_{2}^{4+}$ were highly quenched in water with a weak emission peak centered at 470 nm and an intensity of around $5 \times 10^3$ and $9 \times 10^3$ counts/sec, respectively. OPE quenching was assured through partial proton transfer with the surrounding water molecules deactivating rapidly OPE excited state$^{83,182}$. OPE$_{1}^{2-}$ displayed no fluorescence enhancement in the presence of the monomeric proteins except for lysozyme (Figure 6-2A). The weak OPE fluorescence turn-on in the presence of the monomeric lysozyme might be driven by electrostatic interactions between the positively charged protein (Table 6-2) and the negatively charged OPE. OPE$_{1}^{2-}$ displayed larger fluorescence enhancement in the presence of all amyloid aggregates resulting in ADF values superior to 1 (Table 6-3) indicative of positive sensing. These results show that OPE$_{1}^{2-}$ sensing is not affected by the charge or the morphology of the aggregates. Additionally, OPE$_{1}^{2-}$ was characterized by different spectral signatures depending on the aggregates. In the presence of fibrils made of Aβ40 and insulin, OPE$_{1}^{2-}$ excitation peak was red-shifted (375 nm to 394 nm) while its emission peak was broad and blue shifted (447 nm). This bathochromic red shift indicates the formation of J-dimers, as previously described$^{81,220}$. In the presence of the htau oligomers and lysozyme fibrils, OPE fluorescence was also characterized by a bathochromic red shift but OPE emission was now characterized by two distinct features: one sharp peak at 442 nm and a shoulder at around 470 nm. The presence of two spectral features might indicate
two distinct binding modes. The sharper peak might be caused by the formation of more stable J-dimers, while the shoulder at 470 nm might result from more disordered OPE complexes. Finally, in the presence of PHF6 fibrils, OPE$_1^{2-}$ emission was red shifted (510 nm) and his peak was broader which might indicate the formation of H-aggregates or more disordered OPE agglomerates. The formation of H-aggregates in the presence of PHF6 fibrils might be related to a difference in the fibrillar structure. To better understand these different binding modes of OPE$_1^{2-}$, the structure of the amyloid aggregates should be characterized at a molecular-level.

In contrast, OPE$_2^{4+}$ only selectively detected three amyloid aggregates out of five: fibrils made of lysozyme, insulin and Aβ40 (Table 6-3). The lack of detection of htau oligomers and PHF6 fibrils by OPE$_2^{4+}$ might be caused by the size and/or the morphology of the scaffold. Indeed, the small β-sheet scaffold generated by the hexapeptide PHF6 and the globular structure of htau oligomers might not be suitable for optimal binding of the large OPE$_2^{4+}$. Additionally, similar fluorescence intensity and spectral signature were observed in the presence of all three fibrils made of insulin, lysozyme and Aβ, characterized by a sharp emission peak at around 450 nm and a red shifted excitation peak indicating the formation of highly stable OPE$_2^{4+}$ J-dimers. Finally, a weak OPE$_2^{4+}$ fluorescence turn-on was also observed in the presence of monomeric Aβ and monomeric insulin which might be due to electrostatic interactions between the negatively charged proteins and the positively charged OPE, as previously described.$^{82, 220}$
Figure 6-2: OPE$_1^{2-}$ and OPE$_2^{4+}$ fluorescence spectra at 1 µM in the presence of six amyloid proteins (lysozyme, insulin, Aβ40, Ac-PHF6 and hTau) under the monomeric (blue) or aggregated (red) form. In the case of lysozyme, insulin, Aβ40 and PHF6, the aggregated forms corresponded to the fibrillar conformation, while the oligomeric conformation was tested for hTau*. For PHF6, the non-acetylated peptide was used as monomers and the N-acetylated isoform was used to form fibrils. The unincubated recombinant 441 tau protein was used as the monomeric control for hTau oligomers.
To further characterize OPE sensing of amyloid aggregates, we determined both the dissociation constant and the limit of detection (Table 6-3). An efficient amyloid probe needs to have high affinity toward the aggregated conformation which can be characterized by the dissociation constant ($k_d$). As shown in Table 6-3, in the presence of fibrillar aggregates both OPE$_1$$^{2-}$ and OPE$_2$$^{4+}$ present a $k_d$ value in the sub-micro molar range (0.16-0.96 µM) and a Hill coefficient “h” of 1.5 (Figure S1) indicative of positive binding cooperativity. In contrast, the binding of OPE$_1$$^{2-}$ to htau oligomers is characterized by higher $k_d$ (1.23 µM) indicating lower affinity, and larger $h$ coefficient (4.17) showing higher level of cooperativity. The difference in $k_d$ between fibrillar and oligomeric aggregates indicates that OPE$_1$$^{2-}$ binding affinity is affected by the size and morphology of the scaffold.

The limit of detection (LOD) indicating the smallest protein concentration that OPE can detect with high certainty, was also calculated as previously described$^{220}$. LOD values calculated for both OPEs were in the micro or sub-micromolar range (Table 6-3 and Figure S2), with OPE$_2$$^{4+}$ having the lowest LOD. Interestingly OPE$_1$$^{2-}$ presented similar LOD values for lysozyme, insulin and PHF6 (between 0.52-0.85 µg/mL) but the LOD values were much higher for both Aβ40 fibrils (2.24 µg/mL) and hTau oligomers (4.25 µg/mL). These results indicate that OPE$_1$$^{2-}$ is less sensitive to hTau oligomers which is also supported by a lower affinity (higher $k_d$).

Overall, OPE$_1$$^{2-}$ was more efficient at detecting a wide range of amyloid aggregates characterized by different morphology and charge compared to OPE$_2$$^{4+}$. OPE$_1$$^{2-}$ also displayed a fluorescence signature that can be used to distinguish amyloid aggregates (oligomers vs fibrils). This signature indicates different binding modes which is mainly the result of different aggregate morphologies.
6.3.2 OPE’s sensing of a mixture of fibrillar aggregates

OPEs are efficient probes for selectively detecting amyloid aggregates over their monomeric counterparts. As neurodegenerative disorders are often characterized by the co-deposition of amyloid aggregates made of different proteins, we evaluated OPE sensing properties in the presence of a mixture of amyloid fibrils (Figure 6-3). OPE spectral features were summarized in Table 6-4.

Figure 6-3: OPE\(1^{2-}\) (1) and OPE\(2^{4+}\) (2) fluorescence spectra in the presence of a mixture of amyloid aggregates. A. 21 µg/mL Aβ40 fibrils and 21 µg/mL PHF6 fibrils. B. 21 µg/mL Aβ40 fibrils and 90 µg/mL PHF6 fibrils. C. 21 µg/mL Aβ40 fibrils and 21 µg/mL insulin fibrils. OPE fluorescence was analyzed for the sensor alone at 1 µM (black), in the presence of Aβ40 fibrils (blue), PHF6 fibrils (red), insulin fibrils (orange), both Aβ40 and PHF6 fibrils (green), and both Aβ40 and insulin fibrils (purple). The excitation (dot line) and emission (solid line) spectra were recorded for both sensors.
Table 6-4: Summary of OPE$_{1^-}$ and OPE$_{2^{4+}}$ spectral features observed at 1 µM when incubated alone (OPE background) or in the presence of a mixture of fibrils. A. OPE spectral signature in the presence of 21 µg/mL Aβ40 fibrils and 21 µg/mL PHF6 fibrils. B. OPE spectral signature in the presence of 90 µg/mL Aβ40 fibrils and 21 µg/mL PHF6 fibrils. C. OPE spectral signature in the presence of 21 µg/mL Aβ40 fibrils and 21 µg/mL insulin fibrils.

<table>
<thead>
<tr>
<th>Samples</th>
<th>OPE$_{1^-}$</th>
<th>OPE$_{2^{4+}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emission peak</td>
<td>OPE aggregates</td>
</tr>
<tr>
<td></td>
<td>Peak shape</td>
<td>Peak maximum</td>
</tr>
<tr>
<td>OPE background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 µg/mL Aβ fibrils</td>
<td>Sharp</td>
<td>442 nm</td>
</tr>
<tr>
<td>21 µg/mL PHF6 fibrils</td>
<td>Broad</td>
<td>510 nm</td>
</tr>
<tr>
<td>21 µg/mL Aβ fibrils + 21 µg/mL PHF6 fibrils</td>
<td>Broad</td>
<td>510 nm</td>
</tr>
<tr>
<td>90 µg/mL Aβ fibrils</td>
<td>Sharp</td>
<td>442 nm</td>
</tr>
<tr>
<td>21 µg/mL PHF6 fibrils</td>
<td>Broad</td>
<td>510 nm</td>
</tr>
<tr>
<td>90 µg/mL Aβ fibrils + 21 µg/mL PHF6 fibrils</td>
<td>Sharp</td>
<td>443 nm</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 µg/mL Aβ fibrils</td>
<td>Sharp</td>
<td>442 nm</td>
</tr>
<tr>
<td>21 µg/mL Insulin fibrils</td>
<td>Sharp</td>
<td>442 nm</td>
</tr>
<tr>
<td>21 µg/mL Aβ fibrils + 21 µg/mL Insulin fibrils</td>
<td>Sharp</td>
<td>442 nm</td>
</tr>
</tbody>
</table>

OPE sensing properties were first evaluated in the presence of both Aβ40 and PHF6 fibrils (Figure 6-3 A and Figure 6-3 B). OPE$_{1^-}$ was previously shown to selectively detect both amyloid fibrils with similar $k_d$ (Table 6-3) but the fluorescence intensity and spectral signature generated with both aggregates were different: in the presence of PHF6 fibrils, OPE$_{1^-}$ had higher ADF value, its emission peak was broad and red-shifted indicating the formation of H-aggregates; in the presence of Aβ40 fibrils, OPE$_{1^-}$ emission peak was sharper and was blue-shifted characteristic of J-aggregates (Figure 6-2 and Table 6-4). In contrast, OPE$_{2^{4+}}$ only detected Aβ40 fibrils and only weakly bound to PHF6 fibrils as shown by an ADF value of 0.72 (Table 6-3). When both amyloid fibrils were present at equal concentration (Figure 6-3A), OPE$_{1^-}$ and OPE$_{2^{4+}}$ emission intensities were similar to the sum of the
emissions recorded for both aggregates separately ($OPE_1^{-2} \frac{F_{A\beta f,OPE} + F_{PHF6,OPE}}{F_{(A\beta f+PHF6),OPE}} = 0.93$; $OPE_2^{4+} \frac{F_{A\beta f,OPE} + F_{PHF6,OPE}}{F_{(A\beta f+PHF6),OPE}} = 0.93$). OPE$_1^{-2}$ fluorescence signature generated in the presence of both aggregates was similar to the one obtained in the presence of PHF6 fibrils alone with a peak centered at 510 nm (Figure 6-3 A1). Interestingly, when Aβ40 fibrils concentration was four-time PHF6 fibrils (Figure 6-3 B1), OPE$_1^{-2}$ emission was blue shifted and the spectral signature was characterized by a sharp peak at 443 nm and a new shoulder at around 475 nm. Additionally, OPE$_1^{-2}$ emission intensity generated in the presence of both fibrils was only half of the sum of the emission recorded for PHF6 and Aβ fibrils separately ($OPE_1^{-2} \frac{F_{A\beta f,OPE} + F_{PHF6,OPE}}{F_{(A\beta f+PHF6),OPE}} = 0.54$). The low fluorescence intensity in the presence of both fibrils and the presence of a sharp emission peak at 443 nm and a shoulder at 475 nm might be explained by the presence of both H and J-aggregates. These two types of aggregates are characterized by different energy (J-aggregates display higher energy and so higher fluorescence increase) and different life time (J aggregates have shorter lifetime). The presence of J-aggregates in the sample would dominate the fluorescence and so the fluorescence generated by H-aggregates might not be detectable. The contribution of both H- and J-aggregates could be further analyzed through time-resolved fluorescence. In the case of OPE$_2^{4+}$, the emission spectra obtained in the presence of both aggregates were similar to the one generated in the presence of Aβ40 and PHF6 fibrils separately ($OPE_2^{4+} \frac{F_{A\beta f,OPE} + F_{PHF6,OPE}}{F_{(A\beta f+PHF6),OPE}} = 0.93$).

OPE sensing properties were also evaluated in the presence of both Aβ40 and insulin fibrils (Figure 6-3 C). OPE$_1^{-2}$ displayed similar spectral signature in the presence of both fibrils with an emission peak centered at around 442 nm. OPE$_2^{4+}$ also had similar fluorescence spectrum for both aggregates with a sharp emission peak at around 450 nm and a shoulder at
470 nm. Both sensors were able to detect the mixture of Aβ40 fibrils and insulin fibrils with an emission intensity similar to the sum of the emission generated for both fibrils separately

\[
\frac{F_{(A\beta f)OPE}}{F_{(A\beta f+Insulin f)OPE}} = 0.95; \quad \frac{F_{(A\beta f)OPE}}{F_{(A\beta f+Insulin f)OPE}} = 0.71.
\]

Overall, both OPEs detected a mixture of fibrils. OPE\textsubscript{2} presents a single spectral signature for the detection of different fibrils however in the presence of a mixture of aggregates its fluorescence enhancement reflects the amount of each fibrils. OPE\textsubscript{1} presents a distinct spectral signature depending on the aggregates detected. When two aggregates are present in the samples generating different spectral features, OPE\textsubscript{1} can be used to indicate which aggregates is predominant.

6.3.3 Characterization of OPEs background fluorescence in CSF

The search for a diagnostic tool to track amyloid biomarkers in biological fluids such as CSF is where the field of dementia diagnosis is moving toward. To evaluate OPE sensing properties in CSF, we first characterized OPE background fluorescence in healthy CSF at concentration between 100 nM and 1 µM and compared it to its background fluorescence in phosphate buffer (PB) (Figure 6-4A and B). CSF samples used in this study were purchased from Lee Biosolutions and were collected from a pool of healthy patients (≥ 3). This commercialized healthy CSF had an overall protein concentration of 263 µg/mL as defined through Bradford protein concentration assay and contained highly hydrophobic proteins as observed by reverse phase HPLC (Supplementary Figure S4).
Figure 6-4: OPE$_{1}^{2-}$ and OPE$_{2}^{4+}$ background fluorescence in 10 mM PB (A), in healthy CSF (B), in 1X PBS (C) and in the presence of 4.5 g/L of glucose (D). Both sensors were tested at 4 different concentration: 100 nM, 250 nM, 500 nM and 1 µM. The excitation (dashed line) and emission (solid line) spectra were recorded excepted in the presence of PBS where emission was only reported.

As shown Figure 6-4A1 and A2, both OPEs had low background fluorescence in PB at 100 nM which is explained by a rapid quenching of OPE excited singlet state by surrounding
water molecules trough proton transfer. Both OPEs displayed a weak fluorescence turn-on with increase in concentration characterized by a weak and broad emission peak indicative of the formation of dye complexes in a disordered micelle-like structure likely made of H-type aggregates. In CSF, OPE background fluorescence drastically increased by comparison with PB (3 to 7-fold increase for OPE$_1^{2-}$ and 2 to 3-fold increase for OPE$_2^{4+}$). For both sensors, this increase in fluorescence intensity was also accompanied by a sharpening of the emission spectra which might indicate the formation of J-aggregates. OPEs higher fluorescence in CSF could be explained by a non-selective interaction with other biomolecules such as salt, glucose or non-amylloidogenic protein.

To further understand which biomolecules found in CSF is responsible for OPEs fluorescence turn-on, we investigated OPE fluorescence in the presence of salt, glucose and albumin (Figure 6-4 and Figure 6-5). To evaluate the effect of salt on OPE fluorescence, we tested phosphate buffer solution (PBS) containing 137 mM NaCl and 2.7 mM KCl, a comparable salt concentration to what is found in CSF (150 mM Na$^+$, 3 mM K$^+$ and 1.2 mM Ca$^{2+}$). OPE$_1^{2-}$ did not display any increase in fluorescence intensity or change in spectral signature in PBS, indicating that OPE$_1^{2-}$ fluorescence turn-on in CSF is not caused by the presence of salt. In contrast, OPE$_2^{4+}$ display large fluorescence enhancement in PBS at OPE concentration higher than 500 nM which mimics closely the fluorescence spectrum generated in CSF. Overall, the presence of salt might be one of the biomolecules responsible of OPE$_2^{4+}$ fluorescence turn-on in CSF.

Glucose is another molecule found in CSF at a concentration typically close to 4 mM (0.72 mg/mL). To evaluate the interactions of OPE with glucose we tested a high glucose concentration solution (4.5 g/L ~ 24.75 mM), higher than that found in CSF but also a middle
ground for glucose concentrations in cell culture formulations such as in Dulbecco’s Modified Eagle’s Medium high glucose formulation in which OPE$_1$$^2$$^-$ was also tested (Figure S5). In the presence of 4.5 g/L of glucose, OPE$_1$$^2$$^-$ (Figure 6-4 D1) and OPE$_2$$^{4+}$ (Figure 6-4 D2) displayed similar fluorescence intensity and spectral shifts compared to background fluorescence in PB, indicating a lack of interaction of OPEs with glucose.

CSF is also known to be rich in protein (0.1-0.6 g/L). The most predominant protein in CSF is albumin which is characterized by a cylindrical structure with polar outer walls and a hydrophobic central core. Knowing that OPE sensing of amyloid aggregates relies on binding to their hydrophobic β-sheet structure, the hydrophobicity of albumin might lead to non-selective interaction and non-selective fluorescence turn-on. To evaluate the interaction of OPE to albumin we measured OPE fluorescence in the presence of bovine serum albumin (BSA) known to present a similar helical structure than human serum albumin (HSA) and share 76% of sequence identity with the human isoform. For this assay, OPEs fluorescence was measured in the presence of 263 µg/mL of BSA which compare with the protein concentration found in healthy CSF. In the presence of BSA, both sensors displayed enhanced fluorescence similar to that seen in healthy CSF samples (Figure 6-5) suggesting that the main factor leading to OPEs fluorescence turn-on in CSF is probably their interaction with albumin. This interaction to non-amyloidogenic proteins might lead to a poor sensing of amyloid aggregates in CSF. To compare OPEs interactions to BSA with amyloid aggregates, we analyzed their binding constant and limit of detection (Figure S1 and S2). In the presence of BSA, OPE$_1$$^2$$^-$ and OPE$_2$$^{4+}$ were characterized by $k_d$ values in the sub-micromolar range (0.78 ± 0.07 and 0.19 ± 0.06 µM respectively), similar to the $k_d$ calculated in the presence of amyloid aggregates previously described (Table 6-3). Additionally, both sensors had a hill coefficient
close to 2 in the case of BSA and close to 1.5 in the presence of amyloid aggregates, indicating higher cooperativity with BSA. Finally, the LOD for BSA was found to be 1.64 ± 0.2 µg/mL and 3.50 ± 0.6 µg/mL for both OPE$_1^{2-}$ and OPE$_2^{4+}$, respectively. This LOD was higher than the one calculated in the presence of amyloid aggregates (Table 6-3) indicating that both OPEs can detect pathological protein aggregates at much lower concentration than BSA.

Figure 6-5: OPEs fluorescence at 100 nM in the presence of 263 µg/mL BSA and in CSF (containing 263 µg/mL proteins based on Bradford assay). Excitation (dashed line) and emission (solid line) are represented.

Altogether, we found that both OPEs displayed higher fluorescence turn-on in CSF compared to PB. For both OPEs, this fluorescence enhancement can be explained by their interaction to biological molecules more particularly to albumin. Additionally, the presence of salt seems to affect OPE$_2^{4+}$ fluorescence but not OPE$_1^{2-}$. Despite OPEs fluorescence turn-on in CSF, we found that at the low concentration of 100 nM both OPEs present a minimal background fluorescence which should not alter their sensing properties. For this reason, OPE’s sensing properties in CSF were investigated at 100 nM and not at the typical 1 µM.
6.3.4 OPE’s sensing of fibrillar and oligomeric proteins in CSF

OPEs high selectivity, sensitivity and affinity toward amyloid aggregates and their low fluorescence turn-on in CSF at 100 nM make them promising for the detection of pathological protein aggregates in this physiological sample. To assess OPE sensing properties in such complex solution, we evaluated OPE’s detection of 21 µg/mL amyloid aggregates in CSF and compared it to PB.

Previously described amyloid aggregates (fibrils made from insulin, lysozyme, PHF6 and Aβ40, and oligomers made of htau protein) were spiked in CSF and PB. OPE$_{1}^{-}$ and OPE$_{2}^{4+}$ fluorescence spectra were recorded at 100 nM and are reported in Figure 6-6 and Figure 6-7, respectively. The integrated emission areas were calculated and are summarized in Figure S6. The detection of monomeric proteins was not evaluated because of the limited amount of CSF sample which prevented the calculation of the ADF value. Positive sensing of spiked protein was determined by using the protein detection factor or PDF, as described in Equation 2

$$PDF = \frac{F_{(\text{dye+protein})} - F_{(\text{dye in buffer})}}{F_{(\text{dye in buffer})}}$$ (2)

with $F_{(\text{dye in buffer})}$ and $F_{(\text{dye+protein})}$ being the integrated emission intensity of the sensor alone and sensor in the presence of spiked protein, respectively. Similarly to the ADF, positive PDF value (> 1) indicates positive protein detection. PDF values recorded in PB and CSF are summarized in Figure 6-8.
**Figure 6-6:** OPE$_{1,2}$ fluorescence at 100 nM in the presence of 21 ug/mL amyloid aggregates in PB (A and B) and spiked CSF (C and D). Five amyloid aggregates were tested: insulin fibrils, lysozyme fibrils, PHF6 fibrils, Aβ40 fibrils and hTau oligomers. Both the excitation (A and C) and emission spectra (B and D) were recorded.

**Figure 6-7:** OPE$_{4+}$ fluorescence at 100 nM in the presence of 21 ug/mL amyloid aggregates in PB (A and B) and spiked CSF (C and D). Five amyloid aggregates were tested: insulin fibrils, lysozyme fibrils, PHF6 fibrils, Aβ40 fibrils and hTau oligomers. As a control the monomeric Aβ40 was also evaluated. Both the excitation (A and C) and emission spectra (B and D) were recorded.
Figure 6-8: Protein detection factor (PDF) calculated for OPE$_1^{2-}$ (A) and OPE$_2^{4+}$ (B) at 100 nM in the presence of amyloid aggregates (21 µg/mL) in PB (dark gray) or CSF (light gray). As a control, the PDF value was also determined in the presence of Aβ40 monomers for OPE$_2^{4+}$. The red dashed line represents the PDF value equal to 1 used as a threshold to determine positive detection.

OPE$_1^{2-}$ was previously described to detect all amyloid aggregates when tested at 1 µM in PB. At 100 nM OPE$_1^{2-}$ still displayed a selective sensing of all fibrillar proteins in PB (Figure 6-6B) by reaching fluorescence intensities between 5 to 9x10$^3$ counts/sec leading to a PDF value superior to 1 (Figure 6-8A). However, this sensor failed to detect htau oligomers as its PDF value was below 1. The loss of detection of htau oligomers at the low OPE$_1^{2-}$ concentration of 100 nM might be explained by its lower affinity (higher $k_d$) and higher LOD compared to the other fibrillar proteins (Table 6-3). Overall OPE$_1^{2-}$ spectral signature in the presence of all 4 proteins fibrils was similar to what was previously observed when OPE was tested at 1 µM (Figure 6-2). When the amyloid aggregates were spiked in CSF, OPE$_1^{2-}$ fluorescence was similar to its background fluorescence (Figure 6-6C and D) which led to a PDF value close to 0. This lack of amyloid aggregates detection in CSF might be caused by
the presence of albumin for which OPE$_1$ was previously described to interact with high affinity.

The larger cationic OPE$_2^{4+}$ was previously described to detect insulin, lysozyme and Aβ40 fibrils when tested at 1 μM in the presence of 21 μg/mL protein in 10 mM PB (Figure 6-2), but was not able to detect either PHF6 fibrils or htau oligomers. Same sensing pattern was observed when OPE$_2^{4+}$ was used at 100 nM in PB (Figure 6-7A and B). When the amyloid aggregates were spiked in CSF, OPE$_2^{4+}$ maintained its sensing properties for the fibrillar insulin, lysozyme and Aβ40 (PDF > 1) but also gained sensing of PHF6 fibrils (Figure 6-7A and B, Figure 6-8B). This new detection of PHF6 fibrils could be explained by CSF overall hydrophobicity. In such hydrophobic environment OPE$_2^{4+}$ fluorescence quenching by water molecules is attenuated which might lead to stronger fluorescence turn-on when bound to fibrillar PHF6.

The well-known fluorescent probe ThT was also tested at 100 nM in CSF spiked with Aβ40 fibrils at 21 μg/mL (Figure S6). ThT displayed enhanced-fluorescence in the presence of Aβ40 fibrils spiked in CSF compared to CSF background. However, this increase in fluorescence led to a PDF value below 1 (0.72 ± 0.14) indicative of a poor sensing of Aβ40 fibrils in CSF.

Overall, at 100 nM both OPEs detected amyloid aggregates in PB similarly to what was observed when tested at 1 μM. In CSF, OPE$_1$ lost its sensing properties while OPE$_2^{4+}$ was still able to detect fibrillar insulin, lysozyme and Aβ40 and also gained detection of PHF6 fibrils.
With the understanding of background fluorescence of OPE in CSF and with the knowledge of OPEs’ capabilities to detect amyloid aggregates in such complex environment, we evaluated OPEs sensing properties in CSF isolated from two patients diagnosed with dementia after neurological evaluation. As controls we also tested CSF obtained from two women patients in labor and delivery units (L&D controls) as well as two older patients without neuropathology (age-matched controls). All CSF samples were characterized in terms of albumin, phosphorylated tau on threonine 181 (p-tau 181), Aβ40 and Aβ42 concentrations (Table 6-5). Samples isolated from both L&D and age-matched controls contained albumin at concentration between 0.12 and 0.28 mg/mL which is similar to the protein concentration measured from the commercialized healthy CSF previously tested (0.26 mg/mL). Samples isolated from patients with dementia contained higher albumin concentration (up to 0.59 mg/mL) compared to the control samples, which might be due to a compromised blood-brain barrier. They are also characterized by higher p-tau 181, higher Aβ40 and lower Aβ42 content compared to L&D and age-matched controls.

Table 6-5: Protein concentrations in CSF samples isolated from L&D controls, age-matched controls and patients with dementia. Four proteins concentrations are reported: albumin, phosphorylated tau 181, Aβ40, and Aβ42. These concentrations were determined through western blot analysis.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Albumin (mg/mL)</th>
<th>p-tau 181 (ng/mL)</th>
<th>Aβ40 (ng/mL)</th>
<th>Aβ42 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labor &amp; Delivery controls</td>
<td>0.12 - 0.16</td>
<td>0.043 - 0.044</td>
<td>6.58 - 7.09</td>
<td>0.902 - 0.961</td>
</tr>
<tr>
<td>Age-matched controls</td>
<td>0.22 - 0.28</td>
<td>0.039 - 0.047</td>
<td>6.44 - 8.47</td>
<td>0.752 - 1.12</td>
</tr>
<tr>
<td>Dementia</td>
<td>0.14 - 0.59</td>
<td>0.22 - 0.29</td>
<td>8.15 - 10.6</td>
<td>0.205 - 0.219</td>
</tr>
</tbody>
</table>

OPEs fluorescence at 100 nM generated in both L&D and age-matched controls was measured and compared to the fluorescence recorded in the presence of the commercialized...
healthy CSF (control CSF) (Figure 6-9 and Figure S8). OPE\textsubscript{1}\textsuperscript{2−} background fluorescence in control patients was lower than the fluorescence measured in the commercialized CSF (3.5x10\textsuperscript{3} vs 6x10\textsuperscript{3} counts/sec). In the case of OPE\textsubscript{2}\textsuperscript{4+} the opposite trend was observed (5x10\textsuperscript{3} vs 3x10\textsuperscript{3} counts/sec). The variation in OPE’s background fluorescence in different healthy CSF samples by almost 2-fold indicates that other biological molecules present in CSF might also interfere with OPE’s fluorescence.

OPE’s fluorescence was also monitored in CSF isolated from patients with dementia. No significant difference in terms of intensity or spectral signature with either set of controls were observed. A similar observation was made after diluting CSF by 10-fold in MilliQ water to reduce albumin concentration in the sample (Figure S9). ThT fluorescence was also measured at 100 nM in CSF samples isolated from patients with dementia (Figure S7). No significant difference between CSF controls and dementia CSF was observed with ThT.

The lack of detection of amyloid aggregates in dementia CSF by OPE\textsubscript{1}\textsuperscript{2−} and OPE\textsubscript{2}\textsuperscript{4+} might be explained by OPEs limit of detection previously reported to be in the microgram range, while the expected oligomeric Aβ concentration in AD CSF should be in the picogram range\textsuperscript{234}. A second likely explanation for the lack of detection would be the absence of oligomeric protein in CSF samples as the presence of oligomers has not been confirmed in patients with dementia.
Figure 6-9: OPE$_{1}^{2-}$ (A) and OPE$_{2}^{4+}$ (B) fluorescence at 100 nM in PB and CSF isolated from a healthy commercialized CSF (control CSF), from L&D controls, age-matched controls and patients diagnosed with dementia. Both the excitation (dashed line) and emission (solid line) spectra are reported.

6.3.6 Detection of amyloid aggregates in CSF through protein misfolding cyclic amplification (PMCA) assay

The lack of OPE fluorescence enhancement in CSF isolated from demented patient might be due to low oligomer concentration in CSF (pg/mL range) which is below OPE’s LOD (µg/mL range). To overcome this low oligomer concentration, their seeding potency could be exploited to promote the aggregation of monomeric Aβ through the protein misfolding cyclic amplification assay (Aβ-PMCA). This assay was previously introduced by Soto et al., in 2014$^{241}$. In this study they showed that a low concentration of 3 fM of oligomers can be detected through the Aβ-PMCA assay. They used the Aβ-PMCA assay in CSF isolated from patient suffering of AD and found that these samples were characterized by a fast fibrillation when spiked with Aβ42 monomers which was detectable with the fluorescent amyloid probe ThT. Overall, they showed a significant difference in protein fibrillation between AD CSF and control CSF.
As OPEs were previously described to be superior sensors compared to ThT for the detection of amyloid aggregates, we investigated OPE sensing properties for the detection of oligomers in CSF through the Aβ-PMCA. In this study, Aβ-PMCA assay was first evaluated in PB by incubating 50 µM Aβ40 monomers in the presence of 2.6 µM Aβ40 seed protofibrils at 37 °C for 6 days. The potential interference of albumin on the Aβ misfolding amplification was also evaluated by incubating Aβ40 monomers and the seeds in the presence of 300 µg/mL BSA. The Aβ-PMCA assay was finally assessed in CSF samples isolated from one dementia patient and two age-matched controls where 50 µM Aβ40 monomers were spiked before incubation at 37 °C for 6 days. The generation of amyloid fibrils through protein misfolding cycle was analyzed by TEM imaging (Figure 6-10A) and by ThT, OPE$^2$ and OPE$^4$ fluorescence (Figure 6-10B, C and D).
Figure 6-10: Detection of the seeding activity of CSF isolated from age-matched controls and from patients with dementia by Aβ-PMCA through TEM imaging (A) and ThT (B), OPE$_1$ (C) and OPE$_2$ (D) fluorescence. (A) TEM images were taken after 6 days of incubation. (B), (C) and (D). Fluorescence of the three amyloid sensors was measured at 100 nM in the presence of 21 µg/mL protein. Error bars represent standard deviation from duplicate. As control, the seeding activity of Aβ monomers alone (black), Aβ monomers with seed fibrils (dark blue), and Aβ monomers with seed fibrils and BSA (light blue) were monitored. Three CSF samples were also tested: two samples from age-matched control (orange and purple) and one sample from patient with dementia (red). In each CSF sample, Aβ40 monomers were spiked to reach a final concentration of 50 µM.

As shown on TEM images (Figure 6-10A), Aβ monomers incubated alone after 6 days at 37 °C formed very small features which are likely early oligomers but no large fibrils were produced. In the presence of Aβ protofibril seeds, Aβ monomers formed long and mature fibrils. The fast fibrillation in the presence of protofibrillar seed is also supported by ThT, OPE$_1$ and OPE$_2$ fluorescent turn-on observed after only 1 day of incubation (Figure 6-10B,
The effect of BSA on amyloid fibrillation was tested by incubating Aβ monomers with Aβ seed protofibrils in the presence of BSA at 300 µg/mL. As shown on TEM, fibrils were produced in the presence of BSA indicating that the high concentration of albumin found in CSF should not inhibit the seeding potency of the amyloid aggregates. All three sensors were tested for the detection of Aβ fibrils produced in the presence of BSA. Both OPE$_2^{4+}$ and ThT detected Aβ fibrils produced in the absence of BSA, while OPE$_1^{2-}$ exhibited a weaker fluorescence turn-on. OPE$_1^{2-}$ poor detection of Aβ fibrils in the presence of BSA supports the previous observation that OPE$_1^{2-}$ loses amyloid sensing in CSF due to its non-specific binding to albumin. Finally, the seeding potency of CSF isolated from patients diagnosed with dementia was tested and was compared to two CSF samples isolated from age-matched controls. After 6 days of incubation in the presence of 50 µM Aβ monomers, oligomeric like structure were observed in all three CSF samples, more importantly it appeared that more aggregates were present in dementia CSF compared to control samples, however no large fibrillar structure were observed. To further characterize the difference between all three CSF samples, the amount of soluble oligomers would need to be quantify by size exclusion HPLC. Despite a potential difference in the amount of oligomers, none of the amyloid sensors displayed a selective fluorescence turn-on in demented CSF. The slow protein aggregation in demented CSF might be related to a low “seedable” oligomer concentration in the sample. To evaluate this hypothesis, we determined the lowest Aβ40 seed protofibrils concentration capable of generating detectable Aβ40 fibrils through the Aβ-PMCA assay. As shown **Figure 6-11A**, Aβ40 seed protofibrils promoted fibrillation at concentration as low as 1 ng/mL which was detectable by ThT and OPE$_1^{2-}$ but not OPE$_2^{4+}$ (**Figure 6-11B, C and D**). The lack of detection by OPE$_2^{4+}$ would require further analysis as this experiment was only performed...
once. This seeding concentration of 1 ng/mL is still several order of magnitude higher than the oligomer concentration reported in AD CSF (pg/mL range). Additionally, the minimal seeding concentration calculated here is 1,000-fold higher than what was reported by Soto et al. in 2014 (3fM ~ 12.9 fg/mL) where Aβ42 monomers were used to spike CSF. This difference might rely on the lower aggregation potency of Aβ40 compared to Aβ42. To further evaluate the Aβ-PMCA assay for the detection of oligomers in CSF, Aβ42 monomers could be used instead of Aβ40 monomers. Another option would be to further optimize the incubation condition used for the Aβ-PMCA assay as the temperature, pH and agitation have been previously reported to have a significant influence on fibrillation kinetic.
6.4 Conclusion

There is a continued need for the development of sensing technology for early neurological disease diagnosis. Previous work demonstrated OPE as a promising sensor for the detection of fibrillar and oligomeric pathological protein aggregates. In this study we sought to evaluate OPEs sensing capabilities in a biological fluid, CSF. Through spectroscopic assays, imaging techniques, and curve fitting we have characterized and gained an understanding on how
OPE$_{1^{2^{-}}}$ and OPE$_{2^{4^{+}}}$ behave in CSF mainly composed of non-amyloidogenic proteins, salts, and glucose. Both OPEs displayed fluorescence turn-on in CSF likely caused by their non-selective interaction to albumin and salt. Despite this fluorescence enhancement, both OPEs show minimal background fluorescence when used at 100 nM. At such low concentration, OPEs were still able to detect amyloid aggregates in PB but OPE$_{1^{2^{-}}}$ lost sensing activity in CSF while OPE$_{2^{4^{+}}}$ maintained its sensing properties. These two amyloid sensors were also evaluated in CSF samples isolated from patients diagnosed with dementia which resulted in the lack of sensing of pathologically relevant aggregates. The poor performance of OPE is probably due to the low aggregate concentrations found in CSF in AD patients (picogram range) which are several orders of magnitude lower than our calculated limits of detection (microgram range), creating a hurdle for our sensors. To overcome the low oligomer concentration in CSF, we investigated the protein misfolding cyclic amplification assay by spiking CSF with monomeric Aβ40 which should undergo fast fibrillation in the presence of oligomeric seed. This assay was previously described to detect oligomers at concentration as low as 3 fM. Low protein fibrillation was observed CSF isolated from demented patients resulting in a lack of sensing by OPE. This slow fibrillation might indicate that the assay needs further optimization to ensure rapid fibrillation in the presence of low oligomers seed concentration which could be achieved by changing the incubation conditions. Despite poor performance of OPE in CSF, this work will guide future research to further evaluate OPE for the detection of amyloid aggregates in CSF as a diagnostic tool.
6.5 Experimental Methods

6.5.1 Material

Purified synthetic amyloid β-40 (Aβ40) was acquired from peptide 2.0. Bovine insulin, hen egg white lysozyme and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). N-terminus acetylated PHF6 (Ac-PHF6) and non-acetylated PHF6 (NH₃⁺-PHF6) were synthetized through a solid-phase synthetic method as previously described,¹⁰¹ and were purified by reverse phase HPLC. Human derived tau (htau) oligomers were graciously provided by Dr. Kayed Rakez²⁵². Recombinant tau 441 was produced and purified as previously described²⁵². Human cerebrospinal fluid (CSF) sample pooled from ≥ 3 healthy donors was purchased from Lee Biosolutions (Maryland Heights, MO). CSF sample isolated from patients diagnosed with dementia, from patients in labor and delivery units (L&D controls) and from older patients without neuropathology (age-matched controls) were graciously provided by Dr. Gary Rosenberg. All CSF samples were centrifuged at 8,000 rpm for 8 minutes and supernatant was stored -20 °C until use. Dulbecco’s Modified Eagle’s Medium (DMEM) F12 media and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (HCl) was purchased from Merck, (Kenilworth, NJ). Sodium citrate was acquired from Fisher Scientific, (Hampton, NH). Sodium azide, NaCl and dimethyl sulfoxide (DMSO) were obtained from EMD Millipore (Burlington, MA). Tris was purchased from BioRad (Hercules, CA). Phosphate buffer (PB) was prepared with sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrous purchased from Thermo Fisher Scientific (Waltham, MA). Water was purified by MilliQ purification system (Millipore Synergy UV) before use. ThT was acquired from ACROS Organics (Belgium). OPEs were synthesized and purified by previously published procedures¹⁸¹.
6.5.2 Protein solubilization and preparation of amyloid aggregates

Hen egg-white lysozyme (Sigma-Aldrich, St. Louis, MO) was dissolved at 5 mg/mL in 10 mM sodium citrate pH 3 with 0.1 M NaCl. Protein concentration was verified by using absorbance at 280 nm and the coefficient of extinction \((2.63 \text{ L/(g.cm)})^{253}\). Lysozyme protein was then incubated at 70 °C for 48 hours under magnetic stirring to form fibrils. Insulin from bovine pancreas (Sigma-Aldrich, St. Louis, MO) was solubilized in 25 mM HCl at 599 µM and was incubated at 60 °C for 2 days under quiescent conditions. Synthetic Aβ40 (peptide 2.0, Chantilly, VA) was solubilized in DMSO at 50 mg/mL. After sonicating the peptide for 5 minutes with the 550T Ultrasonic Cleaner (VWR International, Radnor, PA) and removing any insoluble particle by centrifugation at 14,000 g for 15 min, Aβ40 was diluted in 40 mM Tris pH 8.0 buffer containing 150 mM NaCl and 0.01 % w/v sodium azide. The protein was then incubated at 37 °C for 23 days under quiescent conditions to ensure protein fibrillation.

Two tau hexapeptide \(^{306}\text{VQIVYK}\)\(^{311}\) (PHF6) were synthesized by a solid-phase method as previously described\(^{101}\): N-terminus acetylated PHF6 (Ac-PHF6) which is prone to fibrillation and non-acetylated PHF6 (NH\(^3\)-PHF6) known to remain monomeric during incubation\(^{101}\). Peptides were purified by reverse phase HPLC (RP-HPLC) using an acetonitrile-water gradient containing 0.1% TFA. Lyophilized peptides were solubilized in MilliQ water to reach 3 mg/mL. The exact peptide concentration was determined by using absorbance at 280 nm and the coefficient of extinction \((\varepsilon_{280\text{nm}} = 1195.9 \text{ cm}^{-1}\text{M}^{-1})^{117}\). MW\text{Ac-PHF6} = 790 g/mol, MW\text{NH3+-PHF6} = 748 g/mol). NH\(^3\)-PHF6 was stored in -70°C until use, while Ac-PHF6 was incubated at 75 °C for 5 days to form fibrils.
6.5.3 Aβ protein misfolding cyclic amplification (Aβ-PMCA) assay

Aβ40 monomers aggregate-free solution was prepared by solubilizing the peptide at 50 mg/mL in DMSO following by sonication for 1 minutes and centrifugation at 14,000 g for 15 min. The supernatant was stored at -70°C until use. Solution of 50 µM Aβ40 monomers was prepared in 50 mM PB and 100 mM NaCl. Aβ40 monomers were either incubated alone or in the presence of 2.6 µM Aβ40 protofibrils or in CSF aliquots at 37 °C for up to 6 days. Aβ40 protofibrils were prepared through sonication of mature fibrils for 10 minutes with the 550T Ultrasonic Cleaner (VWR International, Radnor, PA). These mature fibrils were initially produced after 23 days of incubation of 100 µM Aβ40 monomers at 37 °C in 50 mM PB and 100 mM NaCl.

6.5.4 Fluorescence measurement

Thioflavin T (ThT), OPE1²⁻ and OPE2⁴⁺ were solubilized in MilliQ water at concentration between 100-250 µM. Dyes concentration were determined from their extinction coefficients (MW_{ThT}: 318.85 g/mol and ε_{ThT}412nm in water: 3.6 x 10⁴ M⁻¹cm⁻¹;43 MW_{OPE1₂⁻}: 724.79 g/mol and ε_{OPE1₂⁻}370 nm in water (3.92 ± 0.013) x 10⁴ M⁻¹cm⁻¹; MW_{OPE2₄⁺}: 1002.64 g/mol and ε_{OPE2₄⁺}378nm in water (8.29 ± 0.033) x 10⁴ M⁻¹cm⁻¹). All dyes were sonicated before use with the 550T Ultrasonic Cleaner (VWR International, Radnor, PA) 4 times for 15 seconds to breakdown any aggregates. OPE and ThT fluorescence spectra were recorded in a quartz cuvette (Starna cells Inc., Atascadero, CA) with the PTI QuantaMaster 40 steady state spectrofluorometer (HORIBA Scientific, Edison, NJ) at concentration between 100 nM and 1 µM for OPE or between 1 to 20 µM for ThT, in the presence of biological samples (amyloid proteins, bovine serum albumin, glucose, PBS and CSF). After 30 minutes of incubation at room temperature the emission spectra were recorded at the excitation wavelengths 390, 410
and 440 nm for OPE$_1^{2-}$, OPE$_2^{4+}$ and ThT, respectively. The excitation spectra were recorded at the emission wavelengths of 450, 460 and 480 nm for OPE$_1^{2-}$, OPE$_2^{4+}$ and ThT, respectively. Each fluorescence measurement was done in duplicate.

6.5.5 Binding constant

OPE’s dissociation constant (k$_d$) to protein was determined from a constant protein concentration binding assay$^{211}$. Briefly, OPE’s fluorescence was recorded at concentration between 0 - 2 µM in the presence of 21 µg/mL of protein. The integrated emission area of OPE was calculated and was background subtracted with the corresponding OPE emission spectrum recorded in 10 mM phosphate buffer (PB). The dissociation constant was determined from the plot of emission integrated area versus sensor concentration. K$_d$ were extracted from data fitting to a nonlinear regression curve assuming site specific binding and using a Hill coefficient (h) to account for any binding cooperativity.

6.5.6 Limit of detection

Limit of detection was defined as being the smallest protein concentration that can be detected by OPE with high certainty and was calculated as previously described (Equation 3)$^{220}$. LOD was determined as being three-time OPE’s background standard deviation. The corresponding protein concentration was determined using the slope of the fluorescence signal vs. concentration correlation

$$\text{Concentration LOD} = \frac{3\sigma}{S} \quad (3)$$

where σ is the standard deviation of the dye alone (blank) and S is the slope of the signal vs. concentration correlation$^{220}$. 

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6.5.7  TEM imaging

Amyloid aggregates were imaged by transmission electron microscopy. The proteins were diluted at 21 µg/mL in MilliQ water and were loaded onto a glow discharge grid. After letting the protein adsorb on the grid for 5 minutes, the excess of protein was wicked away and then the grid was stained using 2% uranyl acetate through four consecutive staining steps. The first one consisted in applying the stain for 3 min and the three last staining steps consisted in applying uranyl acetate for 1 min only. Between each staining step, the excess of stain was wicked away. After the last staining step, the grid was air dried for 30 minutes before imaging with the HITACHI HT7700 transmission electron microscope (Hitachi High Technologies Corp., Tokyo, Japan).

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6.6.2  Funding Sources

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6.7 Acknowledgment

We are thankful to Dr. Yanli Tang and Dr. Eunkyung Ji who originally synthesized the OPE compounds. We are also thankful to Dr. Kayed Rakez who graciously provided human derived tau (htau) oligomers and Dr. Gary Rosenberg for providing CSF sample isolated from patients diagnosed with dementia, from patients in labor and delivery units and from older patients without any history of neurological symptoms.

6.8 Supplementary Information

![Figure S1: Binding assay profiles of OPE$_{1^{-2}}$ (A) and OPE$_{2^{4+}}$ (B) to six proteins present at 21 µg/mL (insulin fibrils, lysozyme fibrils, Aβ40 fibrils, PHF6 fibrils, hTau oligomers and BSA). These saturation binding assay curves were obtained by fitting the data to a nonlinear regression assuming site specific binding with binding cooperativity on OriginPro 9. Experiments were performed in duplicates and error bars represent standard deviations. The maximum specific binding (Bmax), the Hill coefficient (h), the dissociation constant (Kd) and the R$^2$ values are summarized in the table (C). OPE has been shown to form superluminescence complexes at concentration above 10 µM leading to fluorescence red-shift and peak sharpening. To isolate OPE/fibrils binding from OPE complexation, the binding assay was performed at OPE concentration bellow 2 µM.](image-url)
**Figure S2**: Linear correlations between fluorescence integrated area of OPE$_1^{2-}$ (A) and OPE$_2^{4+}$ (B) (1 µM) versus protein concentration. Seven proteins were tested: insulin fibrils, lysozyme fibrils, PHF6 fibrils, hTau oligomers, Aβ40 fibrils, Aβ40 monomers, and BSA. Aβ40 monomers was only tested with OPE$_2^{4+}$. Data were fitted to a linear fit on OriginPro. The intercept and slopes values are summarized in the table below.
Figure S3: ThT fluorescence in the presence of a mixture of fibrils. (A) 21 µg/mL Aβ40 fibrils and 21 µg/mL PHF6 fibrils. (B) 21 µg/mL Aβ40 fibrils and 90 µg/mL PHF6 fibrils. (C) 21 µg/mL Aβ40 fibrils and 21 µg/mL insulin fibrils. ThT fluorescence was analyzed for the sensor alone (black), in the presence of Aβ40 fibrils (blue), PHF6 fibrils (red), insulin fibrils (orange), both Aβ40 and PHF6 fibrils (green), both Aβ40 and insulin fibrils (purple). The excitation (dot line) and emission (solid line) were recorded for both sensors. The additive fluorescence in the presence of both fibrils was determined by calculating the following ratio (~ 1 meaning perfect additive fluorescence):

Condition A: ThT $\frac{F_{A\beta f, OPE} + F_{PHF6 f, OPE}}{F_{(A\beta f + PHF6 f), OPE}} = 0.73$

Condition B: ThT $\frac{F_{A\beta f, OPE} + F_{PHF6 f, OPE}}{F_{(A\beta f + PHF6 f), OPE}} = 1.06$

Condition C: ThT $\frac{F_{A\beta f, OPE} + F_{Ins f, OPE}}{F_{(A\beta f + Ins f), OPE}} = 0.80$

Figure S4: Reverse phase chromatogram of healthy CSF. 100 µL CSF were centrifuged (15 min at 14,000 rpm) to remove insoluble species and the supernatant was injected into the Eclipse XDB C18 column (Agilent Technology, Santa Clara, CA) pre-equilibrated at 40 °C with 95% of mobile phase A (water containing 0.1% TFA) and 5% of mobile phase B (acetonitrile containing 0.1% TFA). The proteins were eluted using a 5-100% linear gradient of mobile phase B over 40 min. The absorbance at 215 nm was monitored. The main protein peak was eluted at around 55% of mobile phase B.
Figure S5: OPE$_2^+$ fluorescence (A) and absorbance (B) spectra in the presence of DMEM F12 media and Aβ fibrils. DMEM F12 media is composed of 15 mM HEPES, 0.055 g/L sodium pyruvate, 3.15 g/L glucose, unknown concentration of sodium bicarbonate and phenol red used as a pH indicator. OPE$_2^+$ spectroscopic change was analyzed in the presence of DMEM F12 media. (A) OPE$_2^+$ emission recorded at 1 µM in 100% and 50% media reached an intensity of around $2.5 \times 10^3$ counts/sec similarly to the fluorescence generated in the presence of 21 µg/mL Aβ fibrils (5 µM). OPE1- emission spectrum recorded in the presence of media also present a new spectral signature with a sharp peak at around 440 nm and a shoulder at around 470 nm. This spectral signature indicates the formation of stable OPE complexed likely made of J-aggregates. (B) OPE$_1^+$ absorbance spectrum in the presence of media was characterized by a drastic right shift and sharpening also supporting the formation of J-aggregates.

Figure S6: OPE$_1^{2-}$, OPE$_2^{4+}$ and ThT emission integrated area recorded at 100 nM in PB (A) and CSF (B) spiked with amyloid proteins at 21 µg/mL. Asterisks indicate significant differences with the sensor alone in buffer (PB or CSF) (t-test with a $p$-value $\leq 0.01$).
Figure S7: Thioflavin T background fluorescence spectra in PB, CSF, and in the presence of Aβ40 fibrils. Excitation and emission spectra of a 100 nM, 1 µM, and 20 µM Thioflavin T in PB (A) and CSF (C). (B) and (D) Fluorescence excitation and emission spectra of 100 nM ThT (blue) in PB and CSF solution compared to fluorescence spectra of spiked solutions with 21 µg/mL of Aβ40 in vitro fibrils. (red lines).
Figure S8: OPE$_{1}^{2-}$, OPE$_{2}^{4+}$ and ThT emission integrated area and detection factor in the presence of CSF samples isolated from healthy patients or patients with dementia. Top: Integrated emission intensity of OPEs and ThT displayed as a bar graphs. Grey bar represents the dye (100 nM) in phosphate buffer, the black bar is the dye (100 nM) in purchased healthy CSF. The red, green, and yellow bars are the sensors in CSF from dementia diagnosed patients, labor and delivery controls, and age matched non-dementia diagnosed patients, respectively. Bottom: The protein detection factor (PDF) of OPEs and ThT in CSF samples isolated from healthy or demented patients. The red dashed-line representing the PDF value of 1 used as a threshold to determine positive detection.
Figure S9: OPE$_1$\(^2\) (A) and OPE$_2$\(^{4+}\) (B) fluorescence at 100 nM in CSF samples diluted 10-fold in water. Three CSF samples were tested: CSF from L&D controls, CSF age-matched controls and CSF from patients diagnosed with dementia. Both the excitation (dashed line) and emission (solid line) spectra are reported.
Figure S10: Seeding potency of Aβ40 sonicated fibrils and CSF evaluation through ThT (A), OPE₁⁺⁺ (B) and OPE₂⁺⁺ (C) fluorescence recorded at day 0 and after 1 and 6 days of incubation at 37 °C.
Chapter 7: Controlled and Selective Photo-oxidation of Amyloid-β Fibrils

by Oligomeric p-Phenylene Ethynylenes

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Figure 7-1: TOC graphic
7.1 Abstract

Photodynamic therapy (PDT) is an attractive strategy to clear toxic amyloid aggregates involved in Alzheimer’s disease. One limitation of such a strategy is off-target oxidation which can be lethal for the surrounding cells. We have recently shown that a fluorescent oligo-p-phenylene ethynylene-based sensor (OPE) selectively detects pre-fibrillar and fibrillar aggregates made of disease-relevant proteins such as amyloid-β and α-synuclein. We also demonstrated that the same sensor photosensitizes singlet oxygen species under illumination once bound to a scaffold through the generation of triplet state. The selective sensing and the controllable generation of singlet oxygens make OPE a promising photosensitizer for the selective photo-oxidation of toxic amyloid aggregates. In this paper we characterized OPE’s selective photosensitizing activity and compared that to the well-known photosensitizer methylene blue (MB). Results show that while MB non-selectively photo-oxidized both monomeric and fibrillar conformations of Aβ40, OPE oxidized only Aβ40 fibrils. We further identified that two histidine residues on the Aβ40 fibril surface and a methionine residue located in the fibril core are oxidized by the OPE. Aβ40 fibril oxidation caused their disassembly into shorter β-sheets rich fibrils that display low cell toxicity and could potentially be cleared by endogenous cellular pathways. The investigation of OPE as a selective Aβ aggregate photo-oxidizer with minimal off-target oxidation will contribute toward the development of PDT as a viable strategy to treat neurodegenerative disorders.

Keywords: Alzheimer’s disease, amyloid aggregates, oligo-p-phenylene ethylene, photo-oxidation, fibrils disassembly
7.2 Introduction

The deposition of amyloid plaques made of the amyloid-β (Aβ) peptide is one of the hallmarks of Alzheimer’s disease\textsuperscript{229, 230}. The deposition of Aβ is driven by its abnormal misfolding and aggregation into small oligomers that subsequently grow into large fibrils\textsuperscript{6-8, 213}. The oligomers, which are transient and heterogeneous in nature, are known to be more toxic than the mature fibrils. Mechanism of their toxicity is still unclear but their interactions with cell membrane that lead to membrane destabilization and pore formation have been proposed to cause cell apoptosis\textsuperscript{9, 21, 22}. Aβ fibrils also play a role in neurodegeneration through impairment in axonal transport\textsuperscript{25, 26} or by inducing the aggregation of tau protein and seeding the formation of neurofibrillary tangle\textsuperscript{254}. Additionally, amyloid aggregates are also involved in the rapid and predictable spatiotemporal disease progression through their cell-to-cell transmissibility\textsuperscript{34, 255, 256}. The role of amyloid aggregates in neurodegenerative impairment make their selective degradation and clearance an attractive therapeutic approach.

Therapeutic strategies targeting amyloid aggregates are currently being investigated, including the use of enzymes (neprilysin\textsuperscript{257}, insulin-degrading enzyme\textsuperscript{258, 259} and endothelin-converting enzyme\textsuperscript{260, 261}) or anti-Aβ immunotherapy that stimulates an immune response to produce antibodies against Aβ plaques\textsuperscript{262}. These approaches have several limitations including the non-selective degradation of the native protein which is believed to have important physiological functions\textsuperscript{263, 264}.

Photodynamic therapy (PDT) is a strategy currently used in oncology\textsuperscript{76, 265} and dermatology\textsuperscript{74} through the oxidation of biological molecules, including proteins, lipids and amino acids, that subsequently leads to cell death\textsuperscript{266}. In PDT, a photosensitizer is used to generate singlet oxygens \(^1\text{O}_2\) (photosensitization type II) and/or free radicals
(photosensitization type I) upon light exposure through energy transfer from an excited triplet state. Several photosensitizers have already been investigated for the photo-oxidation of Aβ such as riboflavin, rose bengal, water-soluble porphyrin molecules, flavin-based catalysts and methylene blue (MB). These studies showed that photo-oxidation of Aβ monomers inhibits fibrillization and that photo-oxidized fibrils disassemble into shorter structures displaying lower cell toxicity. MB is one of the main photosensitizers already approved by the FDA in oncology and has been investigated for photo-oxidation of Aβ plaques both in vitro and in vivo. However, MB has an important drawback due to its non-selective binding to negatively charged substrates such as proteins, lipids or nucleic acids that could lead to non-selective oxidation of cellular organelles causing cell apoptosis. The risk of off-target oxidation highlights the need to develop new photosensitizer compounds that are highly selective toward the aggregated conformation of proteins over their native conformers.

We recently showed that a class of novel oligomeric p-phenylene ethynylene compounds (OPEs) selectively bind to and detect β-sheet rich amyloid fibrils. The small and negatively charged OPE1− (Table 7-1) characterized by one repeat unit with carboxyethyl ester end groups and sulfonate terminated side chains detects fibrils made of two model amyloid proteins, insulin and lysozyme, and two disease-relevant proteins, Aβ and α-synuclein. More importantly, OPE1− was also capable of selectively detecting the more toxic, pre-fibrillar aggregates of Aβ42 and α-synuclein. OPE’s superior sensor performance compared to that of the commonly used thioflavin T dye could be attributed to its high sensitivity to fluorescence quenching where the conjugated sensor is quenched in an aqueous solvent and binding to amyloid aggregates reverses quenching and leads to fluorescence recovery or turn-on. The ability to detect a wilder set of protein aggregate
conformations stems from the combination of modes that leads to fluorescence turn-on of the OPEs, including hydrophobic unquenching, backbone planarization, and OPE complexation upon binding to amyloid aggregates.

In addition to sensing, we recently showed that fluorescence recovery of OPE$_1^{2-}$ from binding to cationic detergent cetyltrimethylammonium bromide (CTAB) is accompanied by the generation of the triplet state which subsequently photosensitizes singlet oxygens. Importantly, as the quenched, or unbound state, of OPE$_1^{2-}$ does not have photosensitizing activity, both fluorescence and photosensitizing properties of the compound are controllable, which makes this probe highly promising as a selective and controllable photosensitizer.

In this study, we evaluated the potential of OPE$_1^{2-}$ as a controllable photosensitizer for the selective photo-oxidization of Aβ fibrils over its monomeric counterpart. The well-known, but non-selective, photosensitizer methylene blue (MB) was also studied and compared to OPE$_1^{2-}$. Oxidation of both Aβ monomers and fibrils with light exposure in the presence of OPE$_1^{2-}$ or MB was investigated. Oxidized amino acids on Aβ fibrils were identified, and the effect of fibril oxidation on fibril morphology, secondary structures, and cell toxicity were evaluated.

**Table 7-1: Structures of OPE$_1^{2-}$ and methylene blue (MB)**

<table>
<thead>
<tr>
<th>Molecule name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPE$_1^{2-}$</td>
<td><img src="image" alt="OPE$_1^{2-}$ Structure" /></td>
</tr>
<tr>
<td>MB</td>
<td><img src="image" alt="MB Structure" /></td>
</tr>
</tbody>
</table>

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7.3 Results

Developing a photosensitizer for the selective oxidation of Aβ40 aggregates over the monomeric conformer is essential to avoid off-target oxidation which could cause side effects in a biological environment. In this study, we evaluated OPE$_1^{2-}$ as a selective photo-oxidizer and compared it the well-known photosensitizer MB.

7.3.1 Spectroscopic features of OPE$_1^{2-}$ and MB in the presence of Aβ40 monomers and fibrils

We have previously shown that the photosensitization activity of OPE$_1^{2-}$ is activated by its fluorescence turn-on through complexation with oppositely charged detergent molecules$^{87}$. In this study, we first evaluated absorbance and fluorescence spectral signatures of both OPE$_1^{2-}$ and MB in the presence of monomeric and fibrillar Aβ40.

Aβ40 fibrils were produced by incubating the monomeric peptide at 150 µM in pH 8.0 Tris buffer at 37 °C over 23 days. At pH 8.0, Aβ40 peptide is negatively charged with a net charge of -4.4$^{65}$. The unincubated Aβ does not present any features on TEM images as shown Figure 7-2A indicating that the peptide is most likely monomeric, while incubated peptide formed large cluster of fibrils (Figure 7-2B). These fibrils were previously described to be rich in β-sheet while the monomeric counterpart is mainly composed of random coil$^{220}$.

Figure 7-2: TEM images of Aβ monomers (A) and fibrils (B). Freshly solubilized Aβ monomers do not present any feature on TEM images while after 23 days of incubation Aβ formed large cluster of fibrils.
OPE$_{1-2}^-$ and MB absorbance and emission spectra were analyzed in the presence of different concentrations of Aβ40 monomers and fibrils (Figure 7-3A1-A2 and B1-B2). OPE$_{1-2}^-$ absorbance spectrum is characterized by two main peaks at 316 nm and 368 nm. In the presence of Aβ monomers and fibrils, OPE$_{1-2}^-$ absorbance spectrum was unchanged in terms of peak position and intensity, as previously described (Figure 7-3A)$^{220}$. MB absorbance spectrum characterized by one main peak at 663 nm increased by 14-22% in the presence of both Aβ monomers and fibrils, but its shape and position stayed unchanged.

OPE$_{1-2}^-$ and MB fluorescence was also characterized (Figure 7-3 A3, A4, B3 and B4). At 1µM, OPE$_{1-2}^-$ is quenched in water and in the presence of Aβ40 monomers between 1 to 5 µM no significant change in emission intensity was observed (Figure 7-3 A3). In the presence of fibrillar Aβ40, OPE$_{1-2}^-$ emission increased and blue shifted with fibrils concentration (Figure 7-3 A4) as previously described$^{191, 220}$. This fluorescence enhancement can be attributed to hydrophobic unquenching, backbone planarization and/or OPE$_{1-2}^-$ complexation leading to the formation of J-dimers$^{181, 269-271}$. These results indicate that OPE$_{1-2}^-$ display a selective fluorescence turn-on upon binding to Aβ fibrils which can be used to track the formation of aggregates. MB is also a fluorescent molecule characterized by an emission peak center at 685 nm. In the presence of both monomeric and fibrillar Aβ, MB emission enhanced by 30-50% which could be induced by its interaction to the negatively charge Aβ peptide causing MB planarization and so preventing non-radiative relaxation decay of the photo-excited MB$^{69, 272}$. This observation indicates that MB interacts similarly to both Aβ conformations.
**Figure 7-3:** Absorbance (1 and 2) and fluorescence emission spectra (2 and 3) of OPE$_{12}^{2-}$ (A) and MB (B) at 1 µM in the presence of varying concentrations of Aβ40 monomers or fibrils (0, 1, 3 and 5 µM). OPE$_{12}^{2-}$ displays a selective fluorescence turn-on in the presence of Aβ40 fibrils which is proportional to the peptide concentration. MB exhibits a weak fluorescence turn-on for both monomeric and fibrillar Aβ40.
7.3.2 DNPH dot blot of Aβ40 oxidation

To evaluate the potency of OPE$_{1^{-2}}$ to selectively photo-oxidize Aβ40 fibrils over the monomeric counterpart, the oxidation state of both Aβ40 conformations irradiated in the presence of OPE$_{1^{-2}}$ were characterized and compared to MB. This study consisted in monitoring the carbonyl content of the protein by DNPH dot blot, which increases as a result of protein photo-oxidation$^{273}$. DNPH dot blot was carried out on Aβ40 fibrils and monomers incubated at 5 µM in the dark and under irradiation between 0 to 6 hours in the absence or presence of a photosensitizer at 1 µM (Figure 7-4). Aβ40 monomers and fibrils do not display any change in the carbonyl content after exposition to light in the absence of photosensitizer (Figure 7-4B, columns 1 and 2). DNPH dot blot of Aβ40 fibrils is slightly darker compared to Aβ40 monomers (Figure 7-4B, columns 1 and 2, incubation time 0 hour). This difference could be attributed to a minor oxidation of the fibrils during the 23 days of incubation at 37 °C necessary to produce fibrils, or could be due to non-specific binding of DNPH to the dense and sticky fibrils. In the presence of both photosensitizers and under light irradiation, Aβ40 fibrils are characterized by a large increase in the carbonyl content (Figure 7-4B, column 5 and 8). When Aβ40 fibrils samples were incubated in the dark, no change was observed (Figure 7-4A column 5 and 8), indicating that both OPE$_{1^{-2}}$ and MB are light-controllable photo-oxidizers. Additionally, when Aβ40 monomers were irradiated in the presence of MB, a slight increase in carbonyl content were observed (Figure 7-4B, column 7) which might indicate that MB oxidized both Aβ40 conformations.
Figure 7-4: Results from DNPH dot blot assay of Aβ40 monomers and fibrils (5 µM) in the presence of OPE$_{1}^{2-}$ or MB (1 µM) at different irradiation times. The presence of a dark dot indicates higher carbonyl content. At time 0-hour Aβ fibrils incubated with MB (column B8) present higher carbonyl content than the same protein incubated alone (column B2) which could be due to short light exposure during membrane preparation causing MB photosensitizing activity turn-on.

7.3.3 Mass spectrometry characterization of Aβ40 oxidation

To further characterize the oxidation of Aβ40 monomers and fibrils by OPE$_{1}^{2-}$ and MB, both peptide conformations were analyzed by electrospray ionization mass spectrometry (ESI-MS).

Figure 7-5 shows ESI-MS chromatograms of Aβ40 monomer (25 µM) before and after irradiation in the presence of OPE$_{1}^{2-}$ and MB (1 µM). The analysis of Aβ40 monomers before irradiation (Figure 7-5A) showed the presence of a peptide with a mass to charge ratio (m/z) of 1082 corresponding to the protonated Aβ40 (+ 4 protons). The ESI-MS analysis of Aβ40 monomer obtained after irradiation in the presence of OPE$_{1}^{2-}$ was similar to the one generated from the native Aβ40 monomer (Figure 7-5C), which indicates that the light treatment in the presence of OPE$_{1}^{2-}$ did not induce any change in the protein. In contrast, in the presence of MB, m/z peaks were right shifted (Figure 7-5B) showing an increase in the overall mass of
the monomeric peptide. Several oxidized Aβ40 monomer species were produced after light treatment with MB which was also confirmed by reverse phase HPLC (Figure S1) where the oxidized peptide was eluted earlier in a broad peak revealing the presence of a mixture of more hydrophilic peptides. The oxidation of monomeric Aβ by MB is also supported by a previous study.\textsuperscript{69}

**Figure 7-5**: Mass spectrometry chromatogram of 25 μM Aβ40 monomers non-irradiated (A) and 25 μM Aβ40 monomers irradiated for 4 hours in the presence of 1 μM of MB (B) or OPE\textsubscript{12} (C). Non-irradiated Aβ40 monomer is characterized by a m/z peak at 1082.80 corresponding to Aβ40 with 4 protonation. Similar profile was observed after irradiation in the presence of OPE\textsubscript{12} indicating that OPE did not oxidize the monomeric peptide. After irradiation of Aβ40 in the presence of MB, a mixture of oxidized monomers appeared.
The oxidation of Aβ40 fibrils by both photosensitizers was also characterized by ESI-MS. Fibrils were digested with the Endoproteinase LysC (Figure 7-6A) and Aβ29-40 fragment was analyzed. After irradiating 25 µM Aβ40 fibrils in the presence of MB at 1 µM, Aβ29-40 peak shifted by 16 Da (Figure 7-6C) indicating the addition of an oxygen. The only amino acid that can be photo-oxidized in this fragment is Met35 suggesting that this amino acid was oxidized into a methionine sulfoxide, as previously described. After treatment with OPE$_1$$^{2-}$, similar changes in Aβ29-40 m/z were observed, however the non-oxidized population was still present in the sample (Figure 7-6D) showing that OPE$_1$$^{2-}$ only partially oxidized Met35.
Figure 7-6: Cleavage sites of Endoproteinase LysC in Aβ40 peptide (A) and ESI mass spectrometry chromatograms of Aβ29-40 fragment generated after fibrils incubation at 25 µM in the dark (B) and after 4 hours irradiation in the presence of 1 µM MB (C) or OPE$_{12}^{2-}$ (D).

7.3.4 Amino acid analysis of oxidized Aβ40

Amino acid analysis (AAA) was also carried out on both Aβ40 monomers and fibrils after light exposition in the presence of MB or OPE$_{12}^{2-}$ (Figure 7-7). OPE$_{12}^{2-}$ was previously shown to not oxidize Aβ40 monomers by ESI-MS (Figure 7-5), for this reason only Aβ40 monomer irradiated in the presence of MB was analyzed (Figure 7-7A). Aβ40 fibrils generated before and after irradiation in the presence of both OPE$_{12}^{2-}$ and MB (Figure 7-7B) were also
characterized. Before AAA, Aβ40 peptides were hydrolyzed with 6 N HCl. This treatment is known to destroy partially Met which affect the accuracy of this analysis\textsuperscript{274}. For this reason, no conclusion was made regarding the effect of MB and OPE\textsubscript{1}\textsuperscript{2} on methionine content.

The amino acid analysis shows that after exposing Aβ40 monomers and fibrils to irradiation in the presence of MB, the histidine content was reduced from 3 to 1 (Figure 7-7A and B). Interestingly, Tyrosine content was partially reduced in Aβ40 monomers which is supported by the ESI-MS analysis (Figure 7-5) but stayed unchanged in Aβ40 fibrils after light treatment in the presence of MB. Aβ40 fibrils irradiated in the presence of both OPE\textsubscript{1}\textsuperscript{2} and MB (Figure 7-7B) were characterized by a reduction in His content and no change in Tyr content. With support of ESI-MS results previously described, two histidine, one tyrosine and one methionine were oxidized in Aβ40 monomers by MB and but only two histidine and one methionine were oxidized in Aβ40 fibrils by both MB and OPE\textsubscript{1}\textsuperscript{2}.

The different oxidation pattern observed in monomeric and fibrillar Aβ40 after light treatment with MB might be explained by a difference in the Tyr environment which can be attributed to the three-dimensional structural conformation of Aβ\textsuperscript{275}. The importance of protein secondary structure for Tyr oxidation was previously described in the case of insulin in which Tyr was found non-oxidized in the native form of the protein (hexamer) but was oxidized when insulin was denatured in 8 M urea\textsuperscript{276}.
Figure 7-7: Amino acid analysis of 25 µM Aβ40 monomers (A) and fibrils (B) before and after 4 hours irradiation in the presence of 5 µM of MB or OPE₁²⁻. Amino acid content was determined by normalizing the raw data with the signal generated for phenylalanine knowing that 3 phenylalanine are present in the protein. The x axis describes the type and number of amino acids expected in Aβ40 peptide. Glutamic acid and glutamine could not be differentiated during this analysis and so they appear as Glx. Similarly, aspartic acid and asparagine appear as Asx. Ile content appear to be lower than expected (around 1.2 instead of 2), which might be explained by the poor hydrolysis of the bound Ile-Ile. Red arrows indicate the three amino acids that can be photo-oxidized (Tyr, Met and His). Methionine is partially destroyed during hydrolysis which prevented any conclusion on the effect of MB and OPE₁²⁻ on Met content.

7.3.5 Molecular dynamics analysis of OPE₁²⁻ binding pockets on Aβ40 protofibrils

The localization of the photo-oxidizable amino acids within the Aβ40 peptide and the position of OPE₁²⁻ binding pockets on the Aβ40 protofibrils is essential to understand the photo-oxidation pattern of this photosensitizer.

Protofibril structure made of 24 Aβ9-40 peptides was obtained from the 2LMN¹⁸⁸ protein databank (PDB) file (Figure 7-8A). The first 8 amino acids formed a disordered region, that is why they are not represented in this structure. The structure of a single Aβ9-40 monomer hairpin was extracted from the protofibril PDB structure after energy minimization and equilibration for 100 ns (Figure 7-8B). This screen shot is a representation of the intrinsically disorder monomers.
Aβ contains 5 amino acids that can be photo-oxidized: His6, His13, His14, Tyr10 and Met35 (Figure 7-8). The localization of these amino acids within Aβ monomer and protofibril are shown Figure 7-8B and Figure 7-8C, respectively. All 5 amino acids are freely exposed to the solvent when the protein is monomeric. In the protofibril, Met35 is buried inside the β-sheet core while His13, His14 and Tyr10 are localized at the surface of the protofibrils and His6 is in the disordered N-terminal fragment (not represented on this structure).

OPE$_1^{2-}$ binding pockets on the Aβ40 protofibril were analyzed by molecular dynamics with 12 OPE molecules. 11 out of 12 OPE molecules bound to the protofibrils either as single molecule (pocket # 1, 4 and 5) or as a complex (pockets # 2, 3 and 6) (Figure 7-9A). More specifically six binding pockets were identified, three of them were located on the β-sheet (#2, 3 and 6; Figure 7-9B and C), one was located on the β-turn (#5; Figure 7-9D) and the last two were at the end of the protofibrils (#1 and 4; Figure 7-9B and E). Tyrosine and histidine were located within 4 Å of OPE$_1^{2-}$ in the case of four binding pockets (#2, 3, 5 and 6) which make them highly susceptible for oxidation by singlet oxygen species which are highly reactive and can diffuse through 100-200 Å in a biological system$^{75, 76, 277}$. In contrast, Methionine was buried inside the core of the fibrils which makes it less accessible to singlet oxygen species as the environment of the amino acids play an important role in the efficiency of the photo-oxidation$^{278}$. OPE$_1^{2-}$ cluster located in the binding pocket #4 (extremity of the protofibrils) was the closest to Met35 within a distance of 5.6 Å. The other OPE$_1^{2-}$ clusters were further away from Methionine (between 19.8 – 41.6 Å) which might explain the partial oxidation of Met35 by OPE$_1^{2-}$ previously observed.
**Figure 7-8**: Localization of the oxidizable amino acids in Aβ40 sequence (A), and their special localization within the monomeric (B) and protofibrillar (C) peptide. Methionine is indicated in yellow, Tyrosine is in green and Histidine is blue.

**Figure 7-9**: OPE$^2$-binding pocket in Aβ protofibrils (A). B, C, D and E show different zoom-in of the 6 binding pockets. A binding pocket might correspond to the interaction of a single OPE or a cluster of OPE. Methionine is indicated in yellow, Tyrosine is in green and Histidine is blue.

### 7.3.6 Effect of oxidation on Aβ40 fibril morphology and secondary structure

The effect of fibrils oxidation on their morphology and secondary structure was characterized by TEM imaging and circular dichroism (CD) spectroscopy, respectively. As showed **Figure 7-10A**, untreated Aβ40 fibrils were long and formed large clusters. After irradiation in the absence of photosensitizer, the fibril clusters appeared smaller but were still present (**Figure**...
7-10B). When the fibrils were irradiated with either MB or OPE$_1^2$ they broke down into shorter protofibrils (Figure 7-10C and D).

Fibrils secondary structure was also analyzed. Aβ40 fibrils had a secondary structure rich in β-sheet with a positive and negative CD peaks at 192 nm and 215 nm, respectively, and was not affected by irradiation in the absence of sensitizer (Figure 7-10E). After irradiation in the presence of OPE$_1^2$ or MB (Figure 7-10G and H), no change in the secondary was observed. Overall, these results indicate that photo-oxidation promotes fibrils breakdown but does not alter their secondary structure.

**Figure 7-10**: TEM images (A-D) and circular dichroism spectroscopy (E-G) of Aβ40 fibrils irradiated in the absence and presence of a photosensitizer. A-D: TEM images of 5 µM Aβ40 fibrils incubated in the absence or presence of 1 µM MB or OPE$_1^2$ were taken before (A) after 4 hours of irradiation in the absence and presence of MB or OPE$_1^2$ (B, C and D). E-G: CD spectra were recorded from 50 µM Aβ fibrils incubated in the absence or presence of 10 µM MB or OPE$_1^2$ overtime during light exposition.
7.3.7 Cell toxicity of oxidized $\text{A}\beta40$ fibrils

We have shown that both $\text{OPE}_1^{2-}$ and MB oxidize $\text{A}\beta40$ fibrils to produce short oxidized protofibrils rich in $\beta$-sheet. It is essential to investigate $\text{OPE}_1^{2-}$ and MB intrinsic cell toxicity as well as oxidized fibrils neurotoxicity.

$\text{OPE}_1^{2-}$ and MB cell toxicity was evaluated at different concentrations (between 1-10 $\mu$M). SHSY-5Y neuroblastoma cells were exposed to dark or light for 5 min in the presence of both photosensitizers before being incubated at 37°C, 5% CO$_2$ for 24 hours. After incubation, cell viability was monitored by MTS assay. As shown Figure 7-11A, cells alone were not affected by the light treatment. In the presence of both MB and $\text{OPE}_1^{2-}$ and after dark treatment, cell viability was close to 100% showing that both compounds are not cytotoxic after 24 hours of incubation at concentration as high as 10 $\mu$M. After exposition to light in the presence of MB at concentration higher than 5 $\mu$M, cell viability was lower than the 70% threshold generally considered for cytotoxicity$^{111}$ and the cell viability was significantly different compared to the dark condition (p-value $\leq 0.01$). In contrast, the presence of $\text{OPE}_1^{2-}$ did not affect cell viability even after light treatment. These results indicate that MB is not toxic when kept in the dark but become cytotoxic when exposed to light at concentration higher than 5 $\mu$M which can be associated to the generation of singlet oxygen species. $\text{OPE}_1^{2-}$ is a more controllable photosensitizer as no toxicity was observed when $\text{OPE}_1^{2-}$ was exposed to light in the presence of neuroblastoma cells.

The effect of photo-oxidation on $\text{A}\beta40$ fibrils cell toxicity was also investigated (Figure 7-11B). Fibrils were incubated at 50 $\mu$M in the dark or under irradiation for 4 hours in 10 mM PB pH 7.4 in the absence and presence of 10 $\mu$M $\text{OPE}_1^{2-}$ or MB. After light treatment, fibrils were added to the cells to reach 20 $\mu$M and were incubated for 48 hours at 37 °C, 5%
CO₂ before monitoring cell viability by MTS assay. In the presence of 4 µM MB, cell viability decreased to 20% in both light and dark conditions, which indicates that after a long incubation of 48 hours MB became cytotoxic which was not observed after 24 hours treatment (Figure 7-11A). MB cell toxicity might be associated to the generation of singlet oxygen species when the samples were exposed to sun light during cell preparation which might be enough to active the photosensitizing activity of MB. In contrast, the presence of 4 µM OPE₁²⁻ (non-irradiated and irradiated) did not cause any reduction in cell viability which confirms that OPE is not cytotoxic even after 48 hours treatment. Aβ40 fibrils exposed to dark and light in the absence of photosensitizer were not cytotoxic, however in the presence of irradiated fibrils the cell viability standard deviation was larger (between 95-65%). This large variation might be caused by the formation of cytotoxic protofibrils during the light treatment as the formation of shorter fibrils were observed after irradiation (Figure 7-10). Cells exposed to oxidized fibrils with OPE₁²⁻ displayed similar viability compared to the non-irradiated fibrils indicating that the shorter oxidized fibrils are not cytotoxic.
Figure 7-11: (A) OPE$_1^{2-}$ and MB cell toxicity after irradiating the cells for 5 min in the presence of the varying concentration of photosensitizer and after 24 hours of incubation. (B) Oxidized Aβ40 fibrils cell toxicity after 48 hours treatment. Cell viability was normalized to the negative control, untreated cells. Error bars represent standard deviations of quintuplet experiments. Red dashed line represents 70% viability threshold used to define cytotoxicity. Blue asterisks indicate significant differences between the dark and irradiated incubations (t-test with a p-value ≤ 0.01).

7.4 Discussion

7.4.1 **OPE$_1^{2-}$ selectively and controllably photo-oxidizes Aβ40 fibrils over monomers**

PDT is an attractive method to selectively photo-oxidize amyloid aggregates and promote their disassembly and clearance to treat neurodegenerative disorders such as Alzheimer’s disease. Photosensitizers used for such application need to be highly selective toward the amyloid aggregates to avoid any off-target oxidation. In this study we evaluated the selective photo-oxidation of Aβ40 fibrils by a novel conjugated polyelectrolyte, OPE$_1^{2-}$, and compared it the well-known photosensitizer MB.
DNPH dot-blot (Figure 7-4), ESI-MS (Figure 7-5 and Figure 7-6) and amino acid analysis (Figure 7-7) were used to monitor the oxidation of both Aβ40 monomers and fibrils when exposed to light in the presence of OPE$_{1}^{2-}$ or MB. DNPH dot-blot revealed that OPE$_{1}^{2-}$ is a light-controllable photosensitizer oxidizing selectively Aβ40 fibrils over the monomeric counterpart with minimal off-target oxidation. The selective oxidation of the fibrillar conformation can be explained by the high binding affinity of OPE$_{1}^{2-}$ to the fibrils ($k_d$: $0.70 \pm 0.1 \, \mu M$)$^{220}$, and its low interaction to the monomeric counterpart as shown by no fluorescence turn-on (Figure 7-3B). ESI-MS and amino acid analysis demonstrated that MB oxidized both Aβ40 conformations under irradiation (Figure 7-5 and Figure 7-7A) which makes it less controllable than OPE$_{1}^{2-}$. The non-selective photo-oxidation of both Aβ monomers and fibrils by MB can be attributed to its interaction to the negatively charged peptides under both conformations as MB was previously described to interaction with monomeric Aβ42 with a binding constant of $48.7 \pm 3.6 \, \mu M^{69}$.

7.4.2 OPE$_{1}^{2-}$ photo-oxidizes His13, H14 and Met35 in Aβ40 fibrils

In Aβ40, five amino acids can be photo-oxidized: 3 Histidine (His6, His13, His14), 1 Tyrosine (Tyr10) and 1 Methionine (Met35)$^{273}$. Amino acid analysis (Figure 7-7) and mass spectrometry (Figure 7-6) showed that after light treatment in the presence of OPE$_{1}^{2-}$ and MB, 2 His and 1 Met were oxidized in Aβ40 fibrils. OPE$_{1}^{2-}$ only partially oxidized Met35 which might be due to a lower singlet oxygen quantum yield (MB $\phi_D \sim 0.5$)$^{80}$ and/or by the position of Met35 in the core of the fibrils (Figure 7-8 and Figure 7-9) limiting its accessibility by singlet oxygen species. To better understand the different photosensitizing activity of OPE$_{1}^{2-}$ and MB, singlet oxygen quantum yield should be characterized by luminescence or
photochemical methods with both photosensitizers. Interestingly, Tyr10 was not oxidized by either molecule in the fibrils despite its close location to His13 and His14 which were both oxidized. The low potency of tyrosine to be photo-oxidize can be explained by the lower rate constant, $K$, for $^1O_2$ quenching by Tyrosine compared to Histidine and Methionine ($K_{\text{His}} = 4.6 \times 10^{-7} > K_{\text{Met}} = 1.3 \times 10^{-7} > K_{\text{Tyr}} = 0.2-0.5 \times 10^{-7}$ M$^{-1}$s$^{-1}$). The photo-oxidation of 2His and 1Met by OPE$_1^2$ and MB is supported by previous studies describing Aβ40 fibrils photo-oxidation by a flavin-based catalyst and Iridium (III) Complex where His13, His14 and Met35 were oxidized (Figure 7-12).

![Figure 7-12: Photo-oxidation sites on Aβ40 (A) and plausible products of oxidation (B)](image)

7.4.3 **OPE$_1^2$-photo-sensitization disassembles Aβ fibrils into non-toxic protofibrils**

Aβ40 fibrils are thermodynamic stable which makes their degradation challenging. Photo-oxidation of Aβ40 fibrils reduces their overall hydrophobicity and could promote their breakdown. To better understand the effect of oxidation on fibrils morphology and secondary structure, we analyzed them by TEM imaging and CD spectroscopy (Figure 7-10), respectively. We showed that after being irradiated with either MB or OPE$_1^2$, Aβ40 fibrils breakdown into short protofibrils rich in β-sheet. Fibrils disassembly might be caused by the
oxidation of methionine located in the core of the fibrils, which could be involved in the stabilization of the long fibrils\textsuperscript{284}. As the protofibrils and oligomers are known to be more toxic than fibrils,\textsuperscript{9,21,22,285} we evaluated cell toxicity of the oxidized protofibrils (Figure 7-11). We observed that oxidized protofibrils do not display higher toxicity compared to untreated fibrils indicating that using PDT to break down the fibrils will not generate more toxic species. Overall, the disassembly of long fibrils into shorter oxidized structure by PDT is promising to assure amyloid aggregates clearance.

### 7.5 Conclusion

In this study we investigated the selective photo-oxidation of Aβ40 fibrils over monomers by OPE\textsubscript{1}\textsuperscript{2-} and compared it to MB. We showed that MB oxidizes non-selectively both Aβ conformations while OPE\textsubscript{1}\textsuperscript{2-} selectively oxidizes Aβ fibrils. OPE\textsubscript{1}\textsuperscript{2-} photo-oxidizes fibrils at the position His13, His14 and Met35 through the generation of \textsuperscript{1}O\textsubscript{2} which causes fibrils disassembly into short oxidized protofibrils characterized by low cell toxicity. OPE\textsubscript{1}\textsuperscript{2-} was previously shown to also selectively interact with oligomeric and fibrillar aggregates made of various disease-relevant proteins including Aβ and α-synuclein, which makes OPE\textsubscript{1}\textsuperscript{2-} ideal to target a large range of toxic amyloid aggregates involved in neurodegenerative disorders. Additionally, the selective interaction to pre-fibrillar aggregates makes OPE\textsubscript{1}\textsuperscript{2-} a potential candidate for photo-oxidizing the most toxic species, oligomers, which could be used for early treatment of neurodegenerative diseases before irreversible damages are observed in patients. Finally, OPE\textsubscript{1}\textsuperscript{2-} selective detection of amyloid aggregates and photodynamic activity could lead to the development of a novel theranostic agent.
7.6 Experimental Methods

7.6.1 Material

Synthetic amyloid β-40 (Aβ40) was purchased from Peptide 2.0 (Chantilly, VA). Tris was obtained from BioRad (Hercules, CA). Sodium chloride (NaCl), dimethyl sulfoxide (DMSO), sodium azide, acetonitrile, methanol and hydrochloride acid (HCl) were acquired from EMD Millipore (Burlington, MA). SH-SY5Y neuroblastoma cells, Dulbecco’s Modified Eagle’s Medium (DMEM) F12 media, fetal bovine serum (FBS), 2,4-dinitrophenylhydrazine (DNPH), Trifluoroacetic acid (TFA) and Tween® 20 were obtained from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin (PS) at 10,000 U/mL, AP Rabbit anti-Goat IgG (H+L) secondary antibody and 1-Step NBT-BCIP substrate were purchased from Thermo Fisher (Waltham, MA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased form Promega (Madison, WI). Goat anti-DNP primary antibody was acquired from Bethyl (Montgomery, TX). OPE$_2^-$ was synthesized and purified by previously published procedures$^{181}$. MB was purchased from Avantor (Radnor, PA). 400 mesh copper grids covered by a Formvar/Carbon film (5-10 nm) were obtained from Ted Pella (Redding, CA) and 2% aqueous uranyl acetate was purchased from Electron Microscopy Sciences (Hatfield, PA).

7.6.2 Aβ40 monomers and fibrils preparation

Lyophilized Aβ40 peptide was solubilized in DMSO at 50 mg/mL. After centrifugation at 14,000 rpm for 15 min, the supernatant was removed and stored in a -70 °C freezer. Monomeric Aβ was diluted to 150 µM with a pH 8.0 40 mM Tris buffer containing 150 mM NaCl and 0.01% sodium azide, and incubated at 37 °C for 23 days to form fibrils$^{220}$. Photo-oxidation of both Aβ40 monomers and fibrils by either OPE$_2^-$ or MB was performed by incubating the peptides with the photosensitizer at a molar ratio 5 to 1 or 25 to 1. The mixtures were either
kept in the dark or exposed to light in a photochamber (Luzchem Research Inc.) using 10 LZC 420 lamps (Osram Sylvania, Wilmington, MA) which emit UV light between 350 and 700 nm at 8W per bulb.

7.6.3 Absorbance and fluorescence measurements

OPE$_2^-$ and MB absorbance and emission spectra were recorded at 1 µM in the presence of varying Aβ concentrations (0, 1, 3 and 5 µM) in pH 7.4 10 mM phosphate buffer (PB) after 30 minutes of incubation in the dark at room temperature. Absorbance spectra were obtained with a Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, MA) in a quartz cuvette (PerkinElmer, Waltham, MA). Emission scans were obtained at excitation wavelengths of 390 nm and 660 nm for OPE$_2^-$ and MB, respectively, and were recorded using a PTI QuantaMaster 40 steady state spectrofluorometer (HORIBA Scientific, Edison, NJ) in a quartz cuvette (Starna cells Inc., Atascadero, CA).

7.6.4 DNPH dot blot

0.2 µm PVDF membrane (ThermoFisher, Waltham, MA) was soaked in 100% methanol for 15 seconds, then in water for 5 minutes, and finally in TPBS for 15 min. After incubating Aβ40 monomers or fibrils with the photosensitizer (5 µM protein with 1 µM photosensitizer) in the dark or under illumination for up to 6 hours, samples were blotted onto the membrane (four times 1 µL) and let dry for 15 minutes. DNPH derivatization of protein carbonyl groups was carried out as previously described$^{286}$. Briefly, the membrane was equilibrated in 2.5 N HCl for 5 minutes before transferring to a 20 mM DNPH solution in 2.5 N HCl for 5 minutes. Excess DNPH was then washed away with three 5 mL aliquots of 2.5 N HCl and then 5 mL aliquots of 100% methanol. The membrane was then immuno-stained. The membrane was immersed in the blocking buffer containing 5% nonfat dry milk in pH 7.4 phosphate buffered
saline (PBS) with 0.1% Tween 20, for 24 hours at room temperature. The membrane was then washed six times with the washing buffer made of Tween20-PBS buffer (5 min per wash) before applying the goat anti-DNP primary antibody diluted at 1:10,000 in blocking buffer, for 2 h under agitation in the dark. The membrane was then washed six times with the washing buffer before applying the rabbit anti-goat IgG secondary antibody, alkaline phosphatase (AP) conjugate diluted at 1: 10,000 in blocking buffer, for 2 h under agitation in the dark. The membrane was washed 3 time with the washing buffer and 3 time with PBS before revealing the dot blot with the 1-Step NBT-BCIP substrate. Once the dots appeared, the membrane was rinsed twice with distilled water for 2 minutes under agitation and was dried overnight before imaging the membrane.

7.6.5 Amino acid analysis (AAA)

Samples containing 25 µM Aβ40 and 5 µM photosensitizer were kept in the dark or were irradiated for 4 hours. After light treatment, samples were sent to the Molecular Structural Facility at University of California Davis for amino acid analysis (AAA) using a sodium citrate buffer system. Briefly, this analysis consisted of drying 100 µL of 25 µM peptide samples and performing a liquid phase hydrolysis using 200 µL 6 N HCl containing 1% phenol for 24 hours at 110 °C. After hydrolysis, the protein was dried and added to norleucine, an internal standard, to reach a final volume of 200 µL. The sample was analyzed on a cation-exchange chromatography column using a L-8800 Hitachi analyzer and a post column ninhydrin reaction detection system.

7.6.6 Protein desalting

Both monomeric and fibrillar proteins were desalted using the Amicon Centrifugal Filter (Millipore Sigma, Burlington, MA). 250 µL of peptide at 150 µM was loaded onto the filter to
which 4 mL of PB was added before centrifuging the filter at 3500 rpm for 20 min. After four washing steps, the retentate was collected and volume adjusted to 250 µL. The protein concentration was then determined using the Bradford protein concentration assay (Sigma-Aldrich, St. Louis, MO).

7.6.7 **Electrospray ionization mass spectrometry (ESI-MS)**

Desalted Aβ40 monomers and fibrils samples were analyzed by ESI-MS after 4 hours of irradiation in the absence and presence of MB or OPE$_1^{2-}$ (25 µM protein with 1 µM photosensitizer). Before analysis, Aβ40 fibrils were digested using Endoproteinase Lys C (New England BioLabs, Ipswich, MA) at 1/50 (w/w) enzyme to protein ratio. The digestion was performed by incubating the sample at 37 °C for 16 hours. Aβ40 monomers and fragmented fibrils were diluted to 5 µg/mL using acetonitrile with 1% TFA and were analyzed under continuous ESI-MS spray on SYNAPT G2 Mass Spectrometry instrument from Waters at the Center for Integrated Nanotechnology (CINT) facility in Los Alamos National Laboratory. This analysis was performed in a positive mode by using the following settings: Capillary = 3.5 kV, sampling cone = 251, extraction cone = 5, source temperature = 120 °C, desolvation temperature = 300 °C, and desolvation gas flow = 650 L/h. Data were analyzed with the software MassLynxV4.1.

7.6.8 **Reverse phase high performance liquid chromatography (RP-HPLC)**

The monomeric protein was analyzed by RP-HPLC on an Agilent 1100 instrument (Agilent Technology, Santa Clara, CA) before and after irradiation in the presence of OPE$_1^{2-}$ and MB (5 µM protein with 1 µM photosensitizer). 110 µL of 5 µM protein was centrifuged at 14,000 rpm for 15 minutes. The supernatant (100 µL) was loaded onto an Eclipse XDB C18 column (Agilent Technology, Santa Clara, CA) pre-equilibrated at 40 °C with 95% of mobile phase A
(water containing 0.1% TFA) and 5% of mobile phase B (acetonitrile containing 0.1% TFA). Aβ40 was eluted using a 5-100% linear gradient of mobile phase B over 40 min. The absorbance at 215 nm was monitored. Each chromatogram was background subtracted using the Agilent ChemStation software.

7.6.9 Circular dichroism (CD) spectroscopy

Desalted protein solutions were diluted to 50 µM in 10 mM PB in the absence or presence of 10 µM MB or OPE\textsubscript{1,2}. After exposition to light, the peptide was loaded into a quartz cuvette with a path length of 1 mm (Starna cells Inc, Atascadero, CA) and analyzed on an AVIV 410 CD Spectrometer (AVIV, Lakewood, NJ) between 190 and 270 nm using an averaging time of 15 seconds. Three scans were recorded per sample and averaged signal was converted to molar ellipticity\textsuperscript{119}.

7.6.10 TEM imaging

Aβ40 samples were diluted to 5 µM using MilliQ water. After the grids were glow discharged (Harrick Plasma Cleaner, Carson City, NV) for 30 seconds, the sample was loaded and let adsorbed for 5 min. After wicking away excess sample, the grid was stained one time for 3 minutes and three times for 1-minute each using 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA). Excess stain was wicked away in between the steps. The grid was then air dried for 30 minutes and imaged using a HITACHI HT7700 transmission electron microscope (Hitachi High Technologies Corp., Tokyo, Japan) with the beam current at 8.0 µA and the accelerating voltage at 80 keV.

7.6.11 Cell toxicity assay

SHSY-5Y cells were cultivated in DMEM media containing 1% PS and 10% FBS at 37°C and 5% CO\textsubscript{2}. When the neuroblastoma cells reached 80% confluency, they were used to set up 96
well plates with 20,000 cells/100 µL well. After 16 hours of incubation, media was changed with serum deprived DMEM media and cells were further incubated for 24 hours to ensure cell synchronization. Cells were then treated with a sample containing appropriate protein and sensitizer concentrations and were incubated for another 24 or 48 hours after which cell viability was monitored by MTS assay. The assay involved adding 20 µL MTS reagent in each well already containing 100 µL media and incubating the plate for 3 hours before measuring the absorbance at 490 nm with a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). The background absorbance of MTS in media alone was subtracted from the absorbance obtained in the presence of cells. Absorbance obtained for untreated cells were also obtained and used as 100% viability.

7.6.12 All atom molecular dynamics (MD) simulation

The initial configurations for the Aβ fibril - OPE$_1^2$- simulations were built with UCSF Chimera$^{287}$. The fibril structure, 2LMN$^{188}$ was obtained from the Protein Databank. In this work, we prepared an Aβ(9-40) protofibril made of 24 peptides to evaluate OPE$_1^2$- binding sites. 12 OPEs were positioned around the protofibril at a distance of 10 Å away from the protofibril surface. The anionic OPE$_1^2$- was built using the GaussView 5 package and geometry optimizations was carried out with Gaussian 09.$^{288}$ Simulations were prepared using the AMBERTools suite$^{289}$. Parameters for simulating the protein structures were obtained from the AMBER14 force field. OPEs were parameterized using the AMBER generalized force field (GAFF) and partial charges were generated using the R.E.D. server$^{290, 291}$. Each system was solvated in explicit water molecules (using the TIP3 model) and counter ions were added to neutralize the system (a total of 48 Na$^+$ ions). Molecular dynamics was performed using the AMBER MD package as previously described$^{224}$. Coordinates for the single Aβ9-40 monomer
hairpin were extracted from the protofibril PDB structure following energy minimization and equilibration of the monomer peptide. Production MD simulations were carried out for 100 ns. OPE$_1^{2-}$ binding sites on the protofibril surface were analyzed using Chimera to determine the proximity of bound OPEs to oxidizable residues on the protofibrils. Residues within 4 Å of bound OPEs (either single or complexed) were selected and counted. Distances of the oxidizable methionine residues to bound OPEs, which were larger than the 4 Å cutoff, were determined as the center-of-mass distances between methionine residues and bound OPEs.

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Figure S1: Reverse phase HPLC of Aβ40 monomers before and after irradiation in the presence of MB and OPE$_{1}^{2-}$. Aβ40 monomers non-irradiated and irradiated in the presence of OPE$_{1}^{2-}$ display a similar elution time corresponding to 44.6-44.4% acetonitrile. When the peptide is irradiated in the presence of MB, the elution profile of Aβ40 monomers changed. The main peak is eluted earlier (43.5% acetonitrile) which shows that Aβ40 peptide became more hydrophilic. Also, the peak presents a should, is not sharp which show the presence of a several populations of Aβ40... The change in hydrophobicity of Aβ40 monomers after irradiation by MB can be explained by oxidation of the protein.
Chapter 8: Summary and Future Directions

8.1 Summary

The research presented in this dissertation contributed to a better understanding of amyloid membrane-mediated toxicity and helped evaluate a novel fluorescent probe as a selective amyloid sensor and controllable amyloid photo-oxidizer.

In Chapter 3, we characterized the molecular-level changes associated with PHF6 insertion into a lipid membrane as part of its toxicity pathway. By using two isoforms, the non-prone to fibrillation NH$_3^+$-PHF6 and the fibrillation prone Ac-PHF6, the importance of fibrillation potency on membrane interaction and disruption was evaluated. Interestingly, both peptides interacted rapidly with the anionic lipid membrane causing membrane destabilization through lipid extraction and were prone to membrane-mediated fibrillation. The fibrillation potency of the peptide influenced the structural changes associated with the interaction of PHF6 to the lipid membrane as well as the rate at which the membrane destabilization took place. In fact, Ac-PHF6 exhibited a faster membrane disruption which was accompanied by a rapid formation of β-sheet structure on the lipid membrane and was also characterized by a fast membrane-mediated fibrillation. Overall, this work contributed to a broader understanding of tau toxicity mechanism. This characterization could contribute to a significant advancement in the development of novel therapeutic strategies.

The following chapters were focused on evaluating oligomeric $p$-phenylene ethynylene (OPE) as an amyloid sensor and selective photo-oxidizer. In Chapter 4, OPE’s capability to detect fibrillar and pre-fibrillar aggregates made of three disease-relevant proteins, Aβ40, Aβ42 and α-synuclein, was characterized. The negatively charged OPE$_1^{2-}$ and the positively charged OPE$_2^{4+}$ were evaluated and compared to the well-known amyloid sensor ThT, in terms
of amyloid detection factor (ADF), limit of detection, and affinity. Both OPEs displayed higher sensing properties for the detection of fibrillar aggregates compared to ThT in terms of binding affinity and ADF value. Strikingly, OPEs detected pre-fibrillar aggregates made of Aβ42 and α-synuclein proteins that ThT failed to detect. The superior sensing properties of OPEs make them ideal to track the formation of early aggregates (oligomers and protofibrils) and to shine light on the amyloid aggregation mechanism which is still poorly understood.

In Chapter 5, OPE was further characterized as an amyloid probe. Its background fluorescence was analyzed as well as its effect on amyloid aggregation and cell toxicity. We found that OPE formed super-luminescent complexes at concentration higher than 10 µM which limits the selective sensing of amyloid aggregates. When used at the usual 1 µM, OPE background fluorescence was minimal and OPE conserved its sensing properties. Additionally, when co-incubated with Aβ40, OPE did not alter neither the aggregation kinetic nor the aggregates morphology, hydrophobicity and toxicity. Overall, OPE’s high sensitivity, selectivity and affinity toward amyloid aggregates and its low neurotoxicity make it ideal to analyze amyloid aggregation kinetic in vitro and in a cell culture environment through fluorescence-microscopy. This potential application will be discussed in the future directions section.

In Chapter 6, we investigated OPE sensing properties in a physiological sample, cerebrospinal fluid (CSF). This investigation consisted in spiking CSF with five amyloid aggregates: lysozyme fibrils, insulin fibrils, Aβ40 fibrils, PHF6 fibrils and htau oligomers. Interestingly, OPE$_{1}^{2-}$ was superior at detecting amyloid aggregates in phosphate buffer but lost its sensing properties in CSF, while OPE$_{2}^{4+}$ remained sensitive to amyloid aggregates in such complex environment. The loss of OPE$_{1}^{2-}$ sensing properties might be due to its non-selective
interaction to albumin present at high concentration in CSF (around 300 µg/mL). The detection of amyloid aggregates in CSF samples isolated from patient with dementia was also investigated. OPE$_2^{4+}$ did not display selective fluorescence turn-on in dementia CSF compared to healthy CSF. The lack of detection might be due to the limit of detection of OPE$_2^{4+}$ which is in the sub-microgram concentration while aggregates concentration in CSF is expected to be in the picogram range. To overcome the low oligomer concentration in CSF, we investigated the protein misfolding cyclic amplification (PMCA) assay by spiking CSF with monomeric Aβ40 which should undergo fast fibrillation in the presence of oligomeric seed. CSF isolated form patients with dementia did not display large amyloid fibrillation. These results will guide future research to evaluate OPE for the detection of amyloid aggregates in CSF as a diagnostic tool through the optimization of the PMCA assay. Additionally, OPE$_2^{4+}$'s detection of amyloid aggregates in CSF could result in new applications such as the detection of amorphous aggregates in protein-based pharmaceuticals. This last application will be discussed in the future direction section.

Finally, in Chapter 7 we evaluated OPE$_1^{2-}$ as a controllable photo-oxidizer of amyloid fibrils made of Aβ40 peptide and comparing it to the well-known photosensitizer methylene blue (MB). MB was found to photo-oxidize both monomeric and fibrillar peptides. In contrast, OPE selectively photo-oxidized Aβ fibrils over the monomeric counterpart. Three amino acids have been found to be oxidized by OPE$_1^{2-}$, His13 and His14 which are located at the surface of the fibrils and Met 35 which is buried inside the fibrillar structure. As a result of photo-oxidation, Aβ40 fibrils disassembled into short protofibrils characterized by low cell toxicity. Taken together, OPEs selective sensing and controllable photo-sensitization activity could lead
to the development of a novel generation of theranostic molecules for the detection and treatment of neurodegenerative diseases.

### 8.2 Future Directions

The research presented here provides a solid understanding of OPE sensing of a large array of pre-fibrillar and fibrillar amyloid aggregates which open the door to a multitude of applications.

The robustness of OPE$_1^2$ sensing properties when co-incubated with Aβ40 described in Chapter 5 and its binding to early aggregates could be employed to further characterized amyloid aggregation *in vitro* and *ex vivo*. First, OPE could be used in real-time fluorescence microscopy to determine amyloid aggregation kinetic parameters$^{214}$. Secondly, OPE low cell toxicity suggests that this probe could be used to monitor protein aggregation in an *ex vivo* cell culture model. Knowing that Aβ aggregation is accelerated when the peptide interacts with cell membrane, we could also gain a better understanding of this off-pathway aggregation by using OPE fluorescence in cell culture real-time imaging.

The selective photo-oxidation of Aβ40 fibrils by OPE$_1^2$ was well characterized in Chapter 7 in terms of oxidized amino acids and effect on fibrils morphology and cytotoxicity. However, the effect of oxidation on amyloid fibrillation potency and on toxicity pathway has not been investigated. As future work, we propose to evaluate the effect of photo-oxidation on oligomers and protofibrils potency to act as aggregation seeds. Furthermore, one of the main toxicity mechanisms of amyloid proteins has been shown to rely on their interaction to cell membrane. This interaction is mainly driven by electrostatic interactions which might be altered by photo-oxidation. Based on this information, it would be interesting to further
characterize the effect of oxidation on Aβ40 interaction to the negatively charged lipid membrane and evaluate the effect on membrane-mediated fibrillation. This additional study could reveal the potential of photo-oxidation not only to breakdown existing fibrils but also to inhibit aggregation and decrease amyloid toxicity.

Finally, OPE sensing of amyloid aggregates in a complex environment such as CSF described in Chapter 6 could lead to other applications such as the detection of amorphous protein aggregates found in protein-based pharmaceutical preparations. The presence of amorphous aggregates is currently a challenge as they can cause total or partial loss of the therapeutic properties or might even be immunogenic\textsuperscript{292}. To evaluate OPE for such application, first OPE sensing of amorphous aggregates should be characterized. Such aggregates could be produced from lysozyme or BSA proteins\textsuperscript{293}. OPE interaction to pharmaceutical proteins such as antibodies should also be investigated. Finally, OPE background fluorescence in the presence of most commonly used excipients (surfactant, amino acids, sugar, polyols, polymers and chelating agent) should be evaluated\textsuperscript{294}. 

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Chapter 9: Abbreviations

AAA, amino acid analysis; Aβ, amyloid β; Aβ-PMCA, Aβ-protein misfolding cyclic amplification; Ac-PHF6, N-terminal acetylated PHF6; AD, Alzheimer’s disease; ADF, amyloid detection factor; BSA, bovine serum albumin; CD, circular dichroism; CSF, cerebrospinal fluid; CTAB, cetyltrimethylammonium bromide; DLS, dynamic light scattering; DMEM, Dulbecco’s Modified Eagle’s Medium; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol; DMSO, dimethyl sulfoxide; DNPH, 2,4-Dinitrophenylhydrazine; ESI-MS, electrospray ionization mass spectrometry; EthD1, ethidium homodimer-1; FBS, fetal bovine serum; FM, Fluorescence microscopy; GAFF, generalized force field; GIXD, grazing incidence X-ray diffraction; h, Hill coefficient; HCl, hydrochloride acid; HEWL, hen egg white lysozyme; HAS, human serum albumin; htau oligomers, human isolated tau oligomers; kd, dissociation constant; LC, liquid condensed; LE, liquid expanded; L&D, labor and delivery units; LOD, limit of detection; LUV, large unilamellar vesicles; MB, methylene blue; MD, molecular dynamics; MTB, microtubule binding; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NaCl, sodium chloride; ND, neurodegenerative disorders; NFTs, neurofibrillary tangles; NH₃⁺-PHF6, PHF6 with a standard N-terminus; NR, Nile red; OPE, oligo-p-phenylene ethynylene; PB, phosphate buffer; PD, Parkinson's disease; PDB, protein databank; PDF, protein detection factor; PDT, photodynamic therapy; PE, phenylene ethynlenes; PET, Positron Emission Tomography; PHF, paired helical filaments; PS, penicillin-streptomycin; POPC, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); RP-HPLC, reverse phase high performance liquid chromatography; SEC, Size exclusion chromatography; TEM, transmission electronmicroscopy;
microscopy; TFA, trifluoroacetic acid; ThT, Thioflavin T; TIRFM, internal reflection fluorescence microscopy; TR-DHPE, Texas Red 1,2-dihexadecanoyl-sn-glycero-3-Phosphoethanoloamine triethylammonium salt; XR, X-ray reflectivity
Chapter 10: References


Imaging and Inhibiting Copper-Induced Cross-Linking of Amyloid Beta Species in Alzheimer's Disease, *Journal of the American Chemical Society* 135, 16397-16409.


conformation-sensitive optical probes for staining and characterization of amyloid deposits, *Chembiochem* 7, 1096-1104.


