Controlled and Selective Photo-oxidation of Amyloid- β Fibrils by Oligomeric *p*-Phenylene Ethynylenes

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Abstract

Photodynamic therapy (PDT) has been explored as a therapeutic strategy to clear toxic amyloid aggregates involved in neurodegenerative disorders such as Alzheimer's disease. A major limitation of PDT is off-target oxidation, which can be lethal for the surrounding cells. We have shown that a novel class of oligo-*p*-phenylene ethynylene-based compounds (OPEs) exhibit selective binding and fluorescence turn-on in the presence of pre-fibrillar and fibrillar aggregates of disease-relevant proteins such as amyloid- β (A β) and α -synuclein. Concomitant with fluorescence turn-on, OPE also photosensitizes singlet oxygen under illumination through the generation of a triplet state, pointing to the potential application of OPEs as photosensitizers in PDT. Herein, we investigated the photosensitizing activity of an anionic OPE for the photo-oxidation of toxic A β aggregates and compared its efficacy to the well-known but non-selective photosensitizer methylene blue (MB). Our results show that while MB photo-oxidized both monomeric and fibrillar conformers of A β 40, OPE oxidized only A β 40 fibrils, targeting two histidine residues on the fibril surface and a methionine residue located in the fibril core. Oxidized fibrils were shorter and more dispersed, but retained the characteristic β -sheet rich fibrillar structure and the ability to seed further fibril growth. Importantly, the oxidized fibrils displayed low toxicity. We have thus discovered a class of novel theranostics for the simultaneous detection and oxidization of amyloid aggregates. Importantly, the selectivity of OPE's photosensitizing activity overcomes the limitation of off-target oxidation of currently available photosensitizers, and represents a significant advancement of PDT as a viable strategy to treat neurodegenerative disorders.

Introduction

Developing photosensitizers that are selective for their intended targets would significantly advance their application in photodynamic therapy (PDT) in the treatment of numerous human diseases as off-target oxidation is a major drawback of current PDT technology.^{1,2} Herein, we evaluate the selective photosensitizing activity of a novel class of conjugated polyelectrolytes, oligo-*p*-phenylene ethynylenes (OPEs), recently developed for detecting the aggregated conformations of amyloid proteins. Demonstration of OPE's selective photo-oxidation of amyloid protein aggregates will enable the development of these compounds in theranostic applications for the simultaneous detection and treatment of protein misfolding diseases, including Alzheimer's and Parkinson's diseases.

A major pathological hallmark of Alzheimer's disease is the deposition of amyloid plaques composed of the amyloid- β (A β) peptide,^{3,4} which results from the abnormal misfolding and aggregation of the peptides into small oligomers that subsequently grow into large fibrils.⁵⁻⁸ The oligomers, which are transient and heterogeneous in nature, are known to be more neurotoxic than the mature fibrils. The mechanism of their toxicity is still unclear but their interactions with cell membranes leading to membrane destabilization and pore formation have been proposed to cause cell apoptosis.⁹⁻¹¹ A β fibrils also play a key role in neurodegeneration through impairment of axonal transport^{12,13} or by inducing the aggregation of tau protein and seeding the formation of neurofibrillary tangles.¹⁴ Additionally, amyloid aggregates are also involved in the rapid and predictable spatiotemporal disease progression through cell-to-cell transmission.¹⁵⁻¹⁷ Because of the central roles amyloid aggregates play in Alzheimer's disease pathogenesis, their selective degradation and clearance is an attractive therapeutic approach.

Therapeutic strategies targeting amyloid aggregates, specifically A β plaques, are currently being investigated and include the use of enzymes (neprilysin¹⁸, insulin-degrading enzyme^{19,20} and endothelin-converting enzyme^{21,22}) and anti-A β immunotherapy.²³ These approaches have several limitations including the non-selective degradation of the native A β peptide which is believed to have important physiological functions.^{24,25}

PDT is a therapeutic strategy currently used in oncology^{26,27} and dermatology.²⁸ In PDT, a photosensitizer is exposed to light, generating singlet oxygen ¹O₂ (photosensitization type II) and/or free radicals (photosensitization type I) through energy transfer from an excited triplet state.²⁹ Those species then oxidize biological molecules, including proteins, lipids and amino

acids, ultimately leading to cell death (e.g., oncology).³⁰ A number of photosensitizers have been investigated for the photo-oxidation of A β including riboflavin,³¹ rose bengal,³² porphyrin-based molecules,^{33,34} flavin-based compounds,^{31,35} polymer³⁶ and carbon nanodots,^{37,38} metal complexes³⁹⁻⁴¹, fullerene-based materials^{42,43} and methylene blue (MB).⁴⁴ These are non-selective and induce photo-oxidation of both A β monomers and aggregates, as well as other biomolecules in the vicinity to the photosensitizers. For example, MB, a major FDA-approved photosensitizer used in oncology^{45,46}, has been investigated for photo-oxidation of A β plaques both *in vitro* and *in vivo*.⁴⁴ Aside from oxidizing both A β monomers and fibrils, MB also non-selectively binds to and oxidize negatively charged proteins, lipids and nucleic acids⁴⁶, leading to cellular apoptosis. These studies show that photo-oxidation of A β monomers inhibits fibrillation³¹⁻³³, which could be beneficial but likely also cause the loss-of-function of the peptide. Encouragingly, photo-oxidation of fibrils has been found to disassemble the fibrils into shorter structures displaying lower cellular toxicity,³⁵ pointing to the potential that PDT targeted at the aggregated, pathogenic conformation of amyloid proteins can be a beneficial therapeutic approach. Several fibril-specific photosensitizers have recently been developed by Kanai et al., including those based on fibril-binding dyes thioflavin-T^{47,48} and curcumin⁴⁹. Importantly, these studies found that photooxidation caused A β aggregate degradation, which attenuated aggregate toxicity and reduced aggregate levels in the brains of AD mouse models.

We recently showed that a class of novel oligomeric *p*-phenylene ethynylene compounds (OPEs) selectively bind to and detect β -sheet rich amyloid fibrils.⁵⁰⁻⁵³ The small and negatively charged OPE₁²⁻ (Table 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,^{50,51} and two disease-relevant proteins, A β and α -synuclein.⁵² More importantly, OPE₁²⁻ is also capable of selectively detecting the more toxic, pre-fibrillar aggregates of A β 42 and α -synuclein.⁵² 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 wherein the conjugated sensor is quenched in an aqueous solvent and binding to amyloid aggregates reverses quenching and leads to fluorescence recovery or turn-on.⁵³⁻⁵⁶ The ability of OPE₁²⁻ to detect a wider set of protein aggregate conformations stems from the combination of different modes that leads to fluorescence turn-on of the OPEs, including hydrophobic unquenching, backbone planarization, and OPE complexation upon binding to amyloid aggregates.^{50,51,55}

In addition to sensing, we recently showed that fluorescence turn-on of OPE_1^{2-} from binding to the cationic detergent cetyltrimethylammonium bromide (CTAB) is accompanied by the generation of a triplet state which subsequently photosensitizes $^1\text{O}_2$.⁵⁷ Importantly, as the unbound (quenched) state of OPE_1^{2-} does not have photosensitizing activity, both fluorescence and photosensitizing properties of the OPE are selective, which makes this probe highly promising as a photosensitizer that is simultaneously controllable with light and selective for the pathogenic conformations of amyloid proteins. As photo-oxidation can potentially reduce the toxicity and promote the clearance of pathogenic amyloid aggregates, the simultaneous sensing and oxidation of the aggregates by OPEs may represent a novel theranostic approach to simultaneously detect and treat protein misfolding diseases.

In this study, we evaluated the potential of OPE_1^{2-} as a selective photo-oxidizer for $\text{A}\beta$ fibrils over its monomeric counterpart and compared its activity to the well-known but non-specific photosensitizer MB. Oxidation of both $\text{A}\beta$ monomers and fibrils with light exposure in the presence of OPE_1^{2-} or MB was characterized. Oxidized amino acids on $\text{A}\beta$ fibrils were identified and quantified, and the effect of fibril oxidation on fibril morphology, secondary structures, cell toxicity, and fibril seeding potency were evaluated.

Table 1: Structures of photosensitizers OPE_1^{2-} and methylene blue (MB) used in this study

Molecule name	Structure
OPE_1^{2-}	
MB	

Results

Spectroscopic features of OPE_1^{2-} and MB in the presence of $\text{A}\beta$ 40 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.⁵⁷ In this

study, we first measured the absorbance and fluorescence spectra of both OPE_1^{2-} and MB in the presence of monomeric or fibrillar $\text{A}\beta_{40}$. $\text{A}\beta_{40}$ fibrils were produced by incubating the monomeric peptide at 150 μM in pH 8.0 40 mM Tris buffer at 37 °C for 23 days. At this pH, the $\text{A}\beta_{40}$ peptide has a net charge of -4.4.⁵⁸ The unincubated $\text{A}\beta$ does not show any features on TEM images (Figure 1A) indicating that the peptide is most likely monomeric, while incubated peptide showed large clusters of fibrils (Figure 1B). These fibrils were previously characterized to be rich in β -sheets while the monomeric peptides are mainly random coils.⁵²

Figure 1: TEM images of $\text{A}\beta$ monomers (A) and fibrils (B). Freshly solubilized $\text{A}\beta$ monomers do not show any features on TEM images while after 23 days of incubation, $\text{A}\beta$ formed large clusters of fibrils. Scale bar = 200 nm.

OPE_1^{2-} and MB absorbance and emission spectra were measured in the presence of different concentrations of $\text{A}\beta_{40}$ monomers and fibrils (Figure 2A1-2A2 and 2B1-2B2). OPE_1^{2-} absorbance spectrum is characterized by two main peaks at 316 and 368 nm. In the presence of $\text{A}\beta$ monomers or fibrils, OPE_1^{2-} absorbance spectra were unchanged in terms of peak positions and intensities, consistent with previous results.⁵² The absorption spectrum of MB, characterized by one main peak at 663 nm, increased by 14-22% in the presence of either $\text{A}\beta$ monomers or fibrils, but the peak shape and position stayed unchanged.

OPE_1^{2-} and MB fluorescence were also characterized (Figure 2A3-2A4 and 2B3-2B4). At 1 μM , OPE_1^{2-} is quenched in water and in the presence of 1 to 5 μM $\text{A}\beta_{40}$ monomers, no significant change in emission intensity was observed (Figure 2A3). However, when added to 5 μM fibrillar $\text{A}\beta_{40}$, OPE_1^{2-} fluorescence emission drastically increased more than 5-fold blue shifted (Figure 2A4). These results indicate that OPE_1^{2-} displays a selective fluorescence turn-on upon binding to $\text{A}\beta_{40}$ fibrils. MB is also a fluorescent molecule characterized by an emission peak centered at 685 nm. In the presence of both monomeric and fibrillar $\text{A}\beta_{40}$, MB emission moderately increased by 30-50% (Figure 2B3-2B4), which could be due to its interaction to the negatively charge $\text{A}\beta$ peptides causing MB to planarize, which prevents non-radiative relaxation decay of the photo-

excited MB.⁴⁴ The similar levels of fluorescence increases indicate that MB interacts similarly to both monomeric and fibrillar A β conformations, making its interaction nonselective.

Figure 2: OPE₁²⁻ displays a selective fluorescence turn-on in the presence of A β 40 fibrils, while MB exhibits weak fluorescence increases in the presence of both monomeric and fibrillar A β 40. Absorbance (1 and 2) and fluorescence emission spectra (3 and 4) of OPE₁²⁻ (A) and MB (B) at 1 μ M in the presence of varying concentrations of A β 40 monomers or fibrils (0, 1, 3 and 5 μ M are shown in black, blue, red and green, respectively).

DNPH dot blot of A β 40 oxidation

Having confirmed that OPE₁²⁻ selectively binds amyloid fibrils over monomeric A β peptides, we then determined if OPE₁²⁻ also only photo-oxidizes A β 40 fibrils and not the monomers. The oxidation states of both A β 40 conformers irradiated in the presence of OPE₁²⁻ were characterized and compared to MB by monitoring the carbonyl content of the peptide using the DNPH (2,4-dinitrophenylhydrazine) dot blot assay.⁵⁹ The assay was carried out on 5 μ M A β 40 fibrils and monomers incubated for up to 6 hours either in the dark or with irradiation in the presence or absence of 1 μ M photosensitizer (Figure 3). In the dark (Figure 3A), no photo-oxidation is detected in A β monomers incubated alone (column 1) or with a photosensitizer (columns 4 and 7) as no dots are visible. A β 40 fibrils showed faint dots (Figure 3A, column 2) that did not become darker over time, indicating a low level of oxidation might have occurred during the 23 days of incubation to produce fibrils. The low intensity dots could also be due to non-specific binding of DNPH to the dense fibrils. Light exposure alone also did not cause any oxidation of A β 40 monomers or fibrils as no changes in their carbonyl contents were observed (Figure 3B, columns 1 and 2). However, in the presence of both photosensitizers and under light irradiation, A β 40 fibrils quickly became oxidized, as evidenced by large increases in carbonyl content where the dots clearly became darker with irradiation time (Figure 3B, columns 5 and 8). Importantly, A β monomers irradiated in the presence of OPE did not become photo-oxidized as no dots were visible (Figure 3B, column 4). Monomers irradiated in the presence of MB showed small increases in carbonyl content as faint dots were visible (Figure 3B, column 7). Taken together, the dot blot assay shows that MB oxidized both monomers and fibrils, although to different extents, and OPE₁²⁻ only oxidized A β fibrils under irradiation.

Figure 3: Results from DNPH dot blot assay show that OPE₁²⁻ selectively oxidized Aβ40 fibrils over monomers under light irradiation while MB non-selectively oxidized both oxidized Aβ40 fibrils and monomers. Aβ monomers (5 μM) and fibrils (5 μM) in the presence of OPE₁²⁻ (1 μM) or MB (1 μM) at different incubation times in the dark (A) or under light irradiation (B). Dots indicate carbonyl groups of oxidized amino acids and the darker the dots, the higher the carbonyl content. Note that at 0 h, Aβ fibrils incubated with MB (column B8) showed a dot, which could be due to short light exposure during membrane preparation causing MB to photosensitize the oxidation of the fibrils.

Mass spectrometry characterization of Aβ40 oxidation

The DNPH dot blot assay qualitatively showed that OPE₁²⁻ selectively induced photo-oxidation of Aβ fibrils, but not monomers. To confirm this selectivity, we turned to molecular techniques of electrospray ionization mass spectrometry (ESI-MS). Figure 4 shows ESI-MS spectra of Aβ40 monomer (25 μM) before and after irradiation in the presence of OPE₁²⁻ or MB (1 μM). A quadruple charged ion (*m/z* of 1082) was observed for the Aβ40 monomer without irradiation (Figure 4A), which corresponds to monomer's expected and deconvolved mass of 4329. The ESI-MS analysis of Aβ40 monomer obtained after irradiation in the presence of OPE₁²⁻ gave results similar to those obtained from the native Aβ40 monomer (Figure 4B), indicating that irradiation in the presence of OPE₁²⁻ did not induce any mass changes in the peptide. In contrast, *m/z* peaks of Aβ monomers irradiated in the presence of MB (Figure 4C) showed a mixture of oxidized species and their corresponding mass increases (oxidation of the following amino acids are suspected: His (155 g/mol) into dehydro-2-imidiazolone derivative (169 g/mol), Met (149 g/mol)

into sulfoxide (165 g/mol), and Tyr (181 g/mol) into 3,4-dihydroxyphenylalanine (197 g/mol))^{49,60,61}). Oxidation of A β monomers was additionally confirmed by reverse phase HPLC (Figure S1) where the oxidized peptide eluted earlier in a broad peak revealing the presence of a mixture of more hydrophilic peptides; this result is consistent with the presence of oxidized peptides that are more hydrophilic. Our results are also consistent with previous reports on MB induced oxidation of monomeric A β .⁴⁴

Figure 4: Mass spectrometry confirms that while MB induces photo-oxidation of A β monomers, OPE₁²⁻ does not. Electrospray ionization mass spectrometry (ESI-MS) chromatograms of 25 μ M of A β 40 monomers (A) and A β 40 monomers irradiated for 4 hours in the presence of 1 μ M of OPE₁²⁻ (B) or MB (C). Non-irradiated A β 40 monomer is characterized by a m/z peak at 1082.80 corresponding to [A β 40 + 4H]⁴⁺. A similar profile was found after irradiation in the presence of OPE₁²⁻ indicating that OPE did not oxidize the monomeric peptide. After irradiation of A β 40 in the presence of MB, a mixture of oxidized monomers with higher m/z values appeared.

The oxidation of A β 40 fibrils by both photosensitizers was also characterized by ESI-MS. Fibrils were first solubilized by enzymatic digestion with Endoproteinase LysC (Figure 5A) and the fragments were separated by HPLC and analyzed with mass spectrometry; out of the three fragments, only the A β 29-40 fragment which contains the oxidizable Met35 residue was well-resolved by mass spectrometry. The expected mass of the native and oxidized A β 29-40 fragments are 1084.6 Da and 1100.6Da, respectively (Figure 5B). The size of these fragments in the presence of the Na adduct is shown in Figure 5B. After irradiating 25 μ M A β 40 fibrils in the presence of 1 μ M MB, all A β 29-40 peaks shifted by 16 Da (Figure 5D) indicating complete oxidation of the fragment. Met35 is likely oxidized as it is the only photo-oxidizable amino acid in this fragment³⁵. After irradiating the fibrils with OPE₁²⁻, oxidized peaks were observed but the non-oxidized fragments were still present in the sample (Figure 5E). OPE₁²⁻ thus only partially oxidized Met35 located in the core of A β fibrils while MB completely oxidized the residue.

A: Endoproteinase LysC cleavage sites

B: Calculated mass of A β 29-40 and oxidized A β 29-40

	(M)	(M+2H) ²⁺	1 Na ⁺ (M+2H) ²⁺	2 Na ⁺ (M+2H) ²⁺	3 Na ⁺ (M+2H) ²⁺
AB29-40	1084.6	543.3	555.3	566.3	577.3
AB29-40 + Met ox.	1100.6	551.3	563.3	574.3	585.3

C: A β 40 fibrils

D: + MB irradiated

E: + OPE₁²⁻ irradiated

Figure 5: Mass spectrometry confirms that both OPE₁²⁻ and MB induces photo-oxidation of Aβ fibrils. A: Cleavage sites of Endoproteinase LysC in Aβ₄₀ peptide as indicated in red. Cleavage product Aβ₂₉₋₄₀ is the only well-resolved fragment from mass spectrometry. In blue is the suspected oxidized methionine in Aβ₂₉₋₄₀ fragment. B: Calculated masses of the Aβ₂₉₋₄₀ fragment with methionine and oxidized methionine. C-E: ESI mass spectrometry chromatograms of the Aβ₂₉₋₄₀ fragment generated from non-irradiated fibrils (C) and after 4 hours of irradiation in the presence of 1 μM MB (D) or OPE₁²⁻ (E).

Amino acid analysis of oxidized A β 40

Amino acid analysis (AAA) was carried out to further confirm A β 40 oxidation as well as to more completely identify oxidized amino acids (Figure 6). A β 40 fibrils before and after light irradiation in the presence of either OPE $_1^{2-}$ or MB were analyzed. As we have confirmed that OPE $_1^{2-}$ does not oxidize A β monomers (Figures 3 and 4), only A β monomers irradiated in the presence of MB was analyzed (Figure 6A). In AAA, A β peptides were cleaved into individual amino acids by hydrolysis using 6 N HCl. Note that Met can become oxidized during acid hydrolysis⁶². For this reason, no conclusion was made regarding the effect of MB and OPE $_1^{2-}$ on methionine content from this analysis.

AAA results show that the His content in A β 40 monomers and fibrils irradiated in the presence of MB reduced from 3 to 1 (Figure 6A and 6B). Thus 2 out of the 3 His residues were oxidized by MB. Interestingly, Tyr content in A β 40 monomers was partially reduced, which is supported by the ESI-MS analysis (Figure 4), but stayed unchanged in A β 40 fibrils after irradiation with MB. A β 40 fibrils irradiated in the presence of OPE $_1^{2-}$ or MB (Figure 6B) showed reduced His content and no change in Tyr content. Combined with ESI-MS results, it can thus be concluded that two His, one Tyr, and one Met residues were oxidized in A β 40 monomers but only two His and one Met residues were oxidized in A β 40 fibrils. The difference is that Tyr is oxidized only in monomers, but not in A β fibrils, which could be due to differences in the residue's solvent exposure in the two different A β conformations. Tyr oxidation has been found to depend on solvent exposure⁶³. For example, Tyr residues in insulin were found to be non-oxidized in the native hexameric conformation but oxidized in an 8 M urea denatured state⁶⁴. Denaturation increases solvent accessibility and thereby the activity of a photosensitizer. In the disordered A β monomer, Tyr is more accessible to MB photosensitization whereas in the fibrillar state, A β fibril has a core that is inaccessible to the solvent, reducing photo-oxidation of Tyr residues by both MB and OPE.

Figure 6: His, Tyr, and Met residues are oxidized in Aβ monomers by MB, while His and Met residues are oxidized in Aβ fibrils by both MB and OPE₁²⁻. Amino acid analysis of 25 μM Aβ40 monomers (A) and fibrils (B) before and after 4-hour irradiation in the presence of 5 μM of MB or OPE₁²⁻. Amino acid content was determined by normalizing the raw data with known signal generated for phenylalanine. The x axis describes the type and number of amino acids expected in a single Aβ40 peptide. Glutamic acid and glutamine could not be differentiated by this analysis and they together appear as Glx. Similarly, aspartic acid and asparagine appear as Asx. Ile content is lower than expected (around 1.2 instead of 2), which might be explained by poor hydrolysis of the Ile-Ile bond. Red arrows indicate the three amino acids that can be photo-oxidized – Tyr, Met and His. Note that as Met can be partially oxidized during hydrolysis, the effect of MB and OPE₁²⁻ on Met content cannot be conclusively made with this method.

Molecular dynamics (MD) simulations of OPE₁²⁻ binding sites on Aβ40 protofibrils

Having confirmed that OPE₁²⁻ selectively oxidized 2 out of 3 His residues and partially oxidized the Met residue on Aβ fibrils, we then sought to understand the photo-oxidation pattern of this photosensitizer by analyzing binding sites of OPE on the fibril and their proximity to oxidizable residues. It is known that singlet oxygen species generated by a photosensitizer can only diffuse through 100-200 Å in a biological system^{27,65,66} and their reactivity additionally depends on their environment such as solvent accessibility.⁶⁷ We utilized all-atom MD simulations to identify OPE binding sites on an Aβ protofibril and analyzed the distances of the oxidizable residues to the bound OPEs.

Aβ40 contains 5 amino acids that can be photo-oxidized: His6, His13, His14, Tyr10 and Met35 (Figure 7A). A protofibril made of 24 Aβ9-40 peptides was obtained from the 2LMN⁶⁸ Protein Data Bank (PDB) file (Figure 7C). The first 8 amino acids remained disordered in the fibril and were not represented in this structure. The structure of a disordered Aβ9-40 monomer was obtained by first removing a peptide from the protofibril PDB structure and then performing energy

minimization and equilibration for 100 ns (Figure 7B). The locations of oxidizable amino acids within the monomer and protofibril are shown in Figure 7B and 7C, respectively. All 4 oxidizable amino acids of A β 9-40 are freely exposed to the solvent when the peptide is monomeric. In the protofibril, Met35 is buried inside the β -sheet core while His13, His14 and Tyr10 are located on the surface of the protofibril. His6 is in the disordered N-terminal region and not represented on these structures of A β 9-40.

OPE₁²⁻ binding sites on the A β 40 protofibril were analyzed by MD with 12 OPE molecules placed around the protofibril. After 100 ns of simulation, 11 out of 12 OPE became bound to the protofibril at six different binding sites either as single OPE molecules (sites 1, 4 and 5) or as OPE complexes (sites 2, 3 and 6) (Figure 8A). Three of the binding sites were located on the β -sheet rich protofibril surface (sites 2, 3 and 6; Figure 8B and C), one was located on the β -turn (site 5; Figure 8D), and the last two were at the ends of the protofibril (sites 1 and 4; Figure 8B and E). Tyr and His residues were located within 4 Å of OPE₁²⁻ in binding sites 2, 3, 5 and 6, which make them highly susceptible for oxidation by singlet oxygen. In contrast, Met was buried inside the core of the fibril which makes it further away from singlet oxygen generated by surface bound OPEs and possibly less prone to oxidation due to its reduced solvent accessibility.⁶⁷ The closest bound OPE₁²⁻ at site 4 on one end of the protofibril was 5.6 Å away from Met35. The other bound OPEs were further away from Met (between 19.8 – 41.6 Å), which might explain the partial oxidation of Met35 observed.

Figure 7: Oxidizable amino acids in the A β 40 sequence (A) and their locations in an A β 9-40 monomer (B) and protofibril (C). Met, Tyr, and His residues are shown in yellow, green, and blue, respectively.

Figure 8: Positions of 12 OPEs (red) placed around an Aβ protofibril after 100 ns of all atom MD simulation (A) show 11 OPEs bound to the protofibril at 6 binding sites. B, C, D, E and F show different zoomed-in views of the 6 binding sites where OPEs bind both as single OPEs or complexes of OPE. Methionine (M), tyrosine (Y) and histidine (H) are shown in yellow, green and blue, respectively. The remainder of the protofibril is shown in ribbon representation with β-sheets colored purple and random coil in white.

Effect of oxidation on Aβ40 fibril morphology and secondary structures

For PDT to be a viable treatment option, the structural and functional properties of the photo-oxidation products need to be characterized to ensure that they will not be harmful. The effect of oxidation on the fibril's morphology and secondary structures were assessed by TEM imaging and circular dichroism (CD) spectroscopy, respectively. As shown in Figure 9A, untreated Aβ40 fibrils were long and in large clusters. After irradiation in the absence of a photosensitizer, the fibril clusters appeared smaller, but were still present (Figure 9B). When the fibrils were irradiated with either MB or OPE₁²⁻, the clusters were largely gone and shorter fibrils were observed (Figure 9C and D).

CD spectra of Aβ40 fibrils showed positive and negative peaks at 192 nm and 215 nm, respectively, indicating a β-sheet rich structure. Irradiation of the fibrils did not affect their secondary structures (Figure 9E). Irradiation in the presence of OPE₁²⁻ or MB (Figure 9G and H) also did not result in significant changes to the fibril's secondary structures; the shorter fibrils remained rich in β-sheets. Taken together, our results indicate that photo-oxidation induced some breakdown of fibrils, but did not alter their β-sheet rich secondary structures.

Figure 9: Photo-oxidation led to some breakdown of fibrils, but did not alter their secondary structures. TEM images and CD spectra of Aβ40 fibrils irradiated in the absence or presence of a photosensitizer. TEM images of 5 μM Aβ40 fibrils: non-irradiated (A), irradiated for 4 hours (B), irradiated in the presence of MB for 4 hours (C), and irradiated in the presence of OPE₁²⁻ for 4 hours (D). CD spectra of 50 μM Aβ fibrils irradiated for various times: irradiated alone (E), irradiated with 10 μM MB (F), and incubated 10 μM OPE₁²⁻ (G).

Effect of oxidation on Aβ40 fibril seeding potency

The ability to seed aggregation is a prominent property of amyloid fibrils as fibril elongation by the addition of monomers is likely the primary aggregation pathway for Aβ40.⁶⁹⁻⁷¹ The effect of oxidation on the seeding potency of Aβ fibrils was evaluated by monitoring Aβ40 monomer (50 μM) aggregation in the presence of 2.6 μM of non-oxidized and photo-oxidized fibril seeds after 72 hours of incubation at 37°C (Figure 10). Fibril seeds were prepared by sonicating mature Aβ40 fibrils in a bath sonicator for 10 minutes (Figure S2).

As shown in the TEM images, Aβ40 monomers formed large clusters of fibrils after 72 hours of incubation in the presence of fibril seeds or fibrils seeds that were irradiated for 4 hours (Figure 10A1 and 10A4). When the seeds were exposed to MB or OPE₁²⁻ in the dark, the seeding potency was not affected as similar mature fibrils were observed after 72 hours of incubation (Figure 10A2 and 10A3). Fibril formation still occurred after incubating the monomers with oxidized seeds, either by MB or OPE. However, the fibrils produced are shorter and no large fibrillar clusters were observed (Figure 10A5 and 10A6). These fibrils were further characterized by CD spectroscopy. As shown in Figure 10B, fibrils produced in the presence of non-oxidized seeds (green) were rich in β-sheet as evidenced by the presence of a negative and positive CD signals at around 195 nm and 218 nm, respectively. Fibrils seeded by oxidized fibrils (MB in red and OPE₁²⁻ in blue) also

contained β -sheets. However, the CD signal was weaker. The percentages of A β 40 monomers remaining after seeded incubation were determined by SEC (Figure 10C). About 18% monomers remained in the samples seeded with non-irradiated and irradiated seeds (Figure 10A1 and A4). Samples incubated with photo-oxidized seeds (Figure 10C: MB: dashed red line and OPE₁²⁻: dashed blue line) contained about 10% monomers after 72 hours of incubation. Taken together, photo-oxidized fibrils did not show reduced seeding potency, but produced fibrils that were shorter and contained less β -sheets.

Figure 10: Oxidized fibrils largely retain the ability to seed A β monomer aggregation. **A:** TEM images of 50 μ M A β 40 monomers incubated with 2.6 μ M non-oxidized (dark) or oxidized (irradiated) fibril seeds for 72 hours at 37 °C. Seeds were prepared by sonicating fibrils treated with MB or OPE₁²⁻ (5 to 1 A β to photosensitizer molar ratio) in the dark or under irradiation for 4 hours. **B:** CD spectra of A β 40 monomers before incubation (black) and after 72 hours of incubation in the presence of non-oxidized fibril seeds (green) or oxidized fibril seeds generated by MB (red) or OPE₁²⁻ (blue). **C:** SE-HPLC chromatograms of A β 40 monomers before incubation (black) and after 72 hours of incubation in the presence of non-irradiated fibril seeds (green) or fibril seeds in the presence of MB (red) or OPE (blue). Fibril seeds were either kept in the dark (solid line) or irradiated for 4 hours (dashed line) before seeding A β 40 monomers. For HPLC, samples were first centrifuged to remove any insoluble aggregates before injecting onto the size exclusion column.

Cell toxicity of oxidized A β 40 fibrils

We have shown that both photo-oxidation by OPE₁²⁻ and MB produced shorter fibrils that are still rich in β -sheets and able to seed further aggregation. It is essential to additionally investigate the effect of oxidation on fibril neurotoxicity as smaller oligomers and protofibrils are widely believed to be more toxic than mature fibrils. Furthermore, whether the photosensitizers are toxic to cells also need to be evaluated.

Toxicity of OPE₁²⁻ and MB on SHSY-5Y neuroblastoma cells was evaluated at different concentrations (1-10 μ M) both in the dark and with a 5-min irradiation. After treatment, cells were incubated at 37°C in 5% CO₂ for 24 hours, after which cell viability was determined by the MTS assay. As shown in Figure 11A, viability of cells was not affected by the light treatment alone. When cells were treated with various concentrations of either MB or OPE₁²⁻ and kept in the dark, cell viability was close to 100% indicating that both compounds were not cytotoxic at concentrations up to 10 μ M. After exposure to light in the presence of varying concentrations of MB, cell viability was lower than the 70% threshold generally considered for cytotoxicity⁷² at MB concentrations higher than 5 μ M. Note that cell viability was significantly lower in the irradiated samples compared to those kept in the dark (p -value ≤ 0.01). These results indicate that MB is not toxic for up to 10 μ M, but becomes cytotoxic when exposed to light at concentrations higher than 5 μ M. This cytotoxicity is likely due to light induced singlet oxygen generation by MB, which induced non-specific oxidation and led to cell death. In contrast, OPE₁²⁻ did not reduce cell viability after light treatment even at 10 μ M. The lack of toxicity indicates that OPE did not exhibit photosensitizing activity in the presence of the neuroblastoma cells. Given that OPE's photosensitizing activity is turned-on by binding, this result also indicates a lack of non-specific binding of the OPE to the cells. Results from this assay point to the potential of the OPE as a selective, in addition to being controllable, photosensitizer with minimal off-target oxidation and toxicity.

The effect of photo-oxidation on A β 40 fibril toxicity was also investigated (Figure 11B). Fibrils were incubated at 50 μ M in the dark or under irradiation for 4 hours in the absence or presence of 10 μ M OPE₁²⁻ or MB. After the 4-hour incubation, fibrils were added to SHSY-5Y cells at 20 μ M with 4 μ M photosensitizer. The cells were then incubated for 48 hours at 37 °C in 5% CO₂ and cell viability was determined by MTS assay. As additional controls, cells were treated with 4 μ M MB or OPE₁²⁻ and incubated for 4 hours in the dark or under irradiation. In the presence of 4 μ M MB,

cell viability decreased by 80% for both dark and light conditions, indicating that MB is cytotoxic with a longer incubation period. Toxicity of MB might be caused by singlet oxygen generation when the samples were exposed to light during preparation. In contrast, the presence of 4 μM OPE_1^{2-} did not cause any reduction in cell viability in both dark and light conditions, which further confirms that the OPE is not cytotoxic even after 48 hours of incubation. Irradiated and non-irradiated A β 40 fibrils were also not cytotoxic. However, the standard deviation of cell viability in the presence of irradiated fibrils was larger (~15% vs. ~8%) which could be caused by the shorter fibrils produced with irradiation. Cells exposed to fibrils oxidized by OPE_1^{2-} displayed similar viability compared to the non-irradiated fibrils indicating that the shorter oxidized fibrils as determined by TEM in Figure 9 were not cytotoxic.

Figure 11: OPE_1^{2-} and oxidized fibrils are not cytotoxic. (A) Viability of SHSY-5Y neuroblastoma cells incubated for 24 hr in the presence of varying concentrations of OPE_1^{2-} or MB in the dark or after 5 min irradiation. (B) Viability of SHSY-5Y neuroblastoma cells incubated for 48 hr in the presence of MB, OPE_1^{2-} or oxidized A β 40 fibrils. In the irradiated condition, samples were exposed to light for 4 hrs prior to adding to SHSY-5Y cells. Cell viability was normalized to the negative control of untreated cells. Error bars represent standard deviations of quintuplet experiments. Red dashed line represents 70% viability threshold commonly used to define cytotoxicity⁷². Blue asterisks indicate significant differences between the dark and irradiated incubations (t-test with a p-value ≤ 0.01).

Interestingly, cells treated with fibrils incubated with MB in the dark showed around 20% viability, comparable with cells treated with just MB. These results are consistent in that fibrils are not toxic and the toxicity in these samples are likely caused by short light exposure during sample preparation that activated MB photosensitizing activity. Surprisingly, cells treated with fibrils irradiated with MB showed a higher ~60% viability. Although the exact cause for this is not known, we hypothesize that the lower toxicity of the irradiated samples is due to MB photo-bleaching or photodegradation during the 4 hours of irradiation, discoloration of the sample was observed after irradiation. Note that in both sets of SHSY-5Y viability experiments, greater than 100% viability was consistently observed when the cells were treated with OPE, although the cause of this increased viability is not yet clear.

Discussion

PDT is an attractive method to photo-oxidize amyloid aggregates, which could promote their disassembly and clearance to treat neurodegenerative disorders such as Alzheimer's disease. The feasibility of such an approach hinges on the discovery of photosensitizers that are selective for the pathogenic, aggregated conformations of amyloid proteins to avoid off-target oxidation that can lead to the loss-of-function of the native amyloid proteins or the death of surrounding cells or tissue. Additionally, the photosensitizer needs to be non-toxic, as do their photo-oxidized products. In this study, we demonstrate that a novel phenylene ethynylene-based oligomer OPE_1^{2-} selectively and controllably photo-oxidizes the fibrils, but not the monomers, of A β 40. The oxidized fibrils retain its β -sheet rich structures and fibril-seeding ability, and are nontoxic.

OPE_1^{2-} selectively and controllably photo-oxidizes A β 40 fibrils over monomers

Three techniques were used to thoroughly evaluate the oxidation of A β 40 monomers and fibrils exposed to light in the presence of OPE_1^{2-} or the well-known, but nonselective MB. DNPH dot-blot (Figure 3), ESI-MS (Figures 4 and 5), and amino acid analysis (Figure 6) results revealed that OPE_1^{2-} is a light-controllable photosensitizer that selectively oxidizes A β 40 fibrils over its 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 ($K_d = 0.70 \pm 0.1 \mu M$)⁵² and its weak interaction with the monomers as shown by a lack of OPE fluorescence turn-on (Figure 2B). In contrast, MB oxidizes both A β 40 monomers and fibrils under irradiation. This lack of selectivity of MB may be attributed to its non-specific interactions to the negatively

charged A β in both conformations as MB has been previously reported to interaction with monomeric A β 42 with a dissociation constant K_d of $48.7 \pm 3.6 \mu\text{M}$.⁴⁴

OPE₁²⁻ photo-oxidizes His13, His14 and Met35 residues in A β 40 fibrils

In A β 40, five amino acids can be photo-oxidized: 3 histidines (His6, His13, His14), 1 tyrosine (Tyr10) and 1 methionine (Met35)⁵⁹. Amino acid analysis (Figure 6) and mass spectrometry (Figure 5) showed that light treatment in the presence of OPE₁²⁻ or MB led to the oxidation of 2 His and 1 Met in A β 40 fibrils. Interestingly, OPE₁²⁻ only partially oxidized Met35 which might be due to a lower singlet oxygen quantum yield compared to that of MB, which is about 0.5.⁴⁶ However, as Met35 is located in the core of the fibril (Figure 8), its accessibility to singlet oxygen and subsequent reaction oxygen species may be reduced compared to fibril-surface exposed His13 and His14. To better understand the difference between the photosensitizing activities of OPE₁²⁻ and MB, their singlet oxygen quantum yields could be characterized by luminescence or photochemical methods⁷³. Interestingly, Tyr10 in fibrils is not oxidized by either photosensitizer despite its close proximity to His13 and His14 which were both oxidized. The low propensity of tyrosine to be photo-oxidized may be due to its lower rate constant (K) for ¹O₂ quenching compared to histidine and methionine ($K_{\text{His}} = 4.6 \times 10^{-7} > K_{\text{Met}} = 1.3 \times 10^{-7} > K_{\text{Tyr}} = 0.2\text{-}0.5 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$)^{67,74}. Moreover, photo-oxidation of His13, His14, and Met35 residues in A β 40 fibrils observed in this study (Figure 12) has also been previously reported in a study where a flavin-based catalyst and an iridium (III) complex were used^{35,75}.

Figure 10: Photo-oxidation sites on A β 40 (A) and plausible products of oxidation (B).

OPE₁²⁻ photo-sensitization disassembles A β fibrils into β -sheet rich, non-toxic, and seeding-competent protofibrils

A β 40 fibrils are highly thermodynamically stable⁷⁶, which makes their degradation challenging. Photo-oxidation of A β 40 fibrils reduces their overall hydrophobicity and could promote their breakdown⁷⁷. To better understand the effect of oxidation on fibril morphology and secondary structures, we analyzed oxidized fibrils by TEM imaging and CD spectroscopy (Figure 9), respectively. Results show that irradiation by either MB or OPE₁²⁻ broke clusters of long A β 40 fibrils into shorter fibrils still rich in β -sheets. Fibril disassembly could be caused by the partial oxidation of Met35 located in the core of the fibril (Figure 7C), which stabilizes the long fibrils⁷⁷. As protofibrils and oligomers are known to be more toxic than fibrils^{9-11,78}, we evaluated cell toxicity of the oxidized fibrils (Figure). We observed that oxidized fibrils do not display higher toxicity compared to non-oxidized fibrils indicating that using PDT to break down the fibrils will not generate more toxic species. Additionally, OPE₁²⁻ is not cytotoxic to cells, even under irradiation, which further supports their use in PDT. The seeding potency of oxidized fibrils was also characterized (Figure C). Oxidized fibril seeds maintained their seeding capacity, however they produced shorter fibrils that were less rich in β -sheet compared to mature non-oxidized fibrils.

Conclusions

Oligomeric conjugated polyelectrolytes such as OPE₁²⁻ have been recently shown to selectively and sensitively bind to aggregated conformations of a number of amyloid proteins over their monomeric conformers.⁵² Concomitant with aggregate binding is the turn-on of OPE's fluorescence and singlet oxygen generation,⁵⁷ suggesting that OPEs are potentially superior photosensitizers for the PDT treatment of protein misfolding diseases such as Alzheimer's disease. In this study, we carry out a proof-of-concept investigation of the controlled and selective photo-oxidation of A β 40 fibrils over monomers by OPE₁²⁻ and compared its photosensitizing activity to MB. We showed that MB non-selectively oxidizes both A β monomers and fibrils, while OPE₁²⁻ only oxidizes A β fibrils. Three amino acids on the fibril are oxidized by OPE photosensitization, His13, His14 and Met35, which proceeds through binding induced generation of ¹O₂. Photo-oxidation causes fibrils to disassemble into shorter, but non-toxic oxidized fibrils. The oxidized fibrils also retain their ability to seed further A β 40 aggregation, albeit fibrils of a lower β -sheet content were produced. Overall, this study demonstrates the ability of OPE₁²⁻ to controllably and

selectively photo-sensitize the oxidation of A β fibrils. The selective nature of OPE's photosensitizing activity overcomes the major drawback of off-target oxidation from using conventional photosensitizers such as MB in PDT. Combined with its selective fluorescent sensing capabilities, our results from this study support the further development of OPEs as potential theranostics for the simultaneous detection and clearance of amyloid aggregates in protein misfolding diseases such as Alzheimer's disease.

Experimental Methods

Materials

Synthetic amyloid- β (1-40) (A β 40) 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 purchased 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 from Promega (Madison, WI). Goat anti-DNP primary antibody was acquired from Bethyl (Montgomery, TX). OPE₁²⁻ was synthesized and purified by previously published procedures⁵⁵. 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).

A β 40 monomers and fibrils preparation

Lyophilized A β 40 peptide was solubilized in DMSO at 50 mg/mL. After centrifugation at 14,000 rpm for 15 min, the supernatant was removed and stored at -70 °C. Monomeric A β was prepared by diluting the stock solution to 150 μ M with a pH 8.0 40 mM Tris buffer containing 150 mM NaCl and 0.01% sodium azide. Fibrils were made by incubating A β monomers at 37 °C for 23 days⁵². Photo-oxidation of both A β 40 monomers and fibrils by either OPE₁²⁻ or MB was performed by incubating the peptides with a photosensitizer at a peptide to photosensitizer molar

ratio of 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 emitted UV light between 350 and 700 nm at 8 W per lamp. Samples were then irradiated for 0-6 hours for the various experiments described below.

Seeding experiment preparation

Stock A β at 50 mg/mL in DMSO was diluted to 50 μ M using 50 mM phosphate buffer (PB) with 100 mM NaCl at pH 7.4. This A β 40 monomer solution was either incubated alone or in the presence of 2.6 μ M A β 40 seed protofibrils at 37 °C for 72 hours. Three different seed protofibrils were prepared from mature fibrils: (1) A β 40 fibrils incubated for 4 hours in the dark or under irradiation, (2) A β 40 fibrils incubated in the presence of OPE $_1^{2-}$ for 4 hours (5 to 1 molar ratio A β to photosensitizer) in the dark or under irradiation and (3) A β 40 fibrils incubated in the presence of MB for 4 hours (5 to 1 molar ratio A β to photosensitizer) in the dark or under irradiation. Irradiation was carried out in a photo-chamber (Luzchem Research, Inc.) containing 10 bulbs (350-750 nm). Seed protofibrils were then prepared by sonicating the above incubated fibrils for 10 minutes using a 550T Ultrasonic Cleaner (VWR International, Radnor, PA). Note that the mature fibrils prepared by 23-day incubation were not processed, i.e., washed or undergone any separation, prior to the 4 hour incubation and sonication to produce seed protofibrils.

Absorbance and fluorescence measurements

OPE $_1^{2-}$ and MB absorbance and emission spectra were recorded at 1 μ M in the presence of varying concentrations of A β 40 (0, 1, 3 and 5 μ M) in pH 7.4 10 mM 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 $_1^{2-}$ 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).

DNPH dot blot

0.2 μ m PVDF membrane (ThermoFisher, Waltham, MA) was soaked in 100% methanol for 15 seconds, then in water for 5 minutes, and finally in pH 7.4 phosphate buffered saline containing 0.1% Tween 20 (TPBS) for 15 min. After incubating A β 40 monomers or fibrils with a photosensitizer (5 μ M protein with 1 μ M photosensitizer) in the dark or under illumination for up to 6 hours, samples were blotted onto the membrane four times at 1 μ L each and let dry for 15 minutes. DNPH derivatization of protein carbonyl groups was carried out as previously described⁷⁹. 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 of 100% methanol. The membrane was then immuno-stained. First the membrane was submerged in the blocking buffer (pH 7.4 phosphate buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20) for 24 hours at room temperature. The membrane was then washed six times with a washing buffer (PBS containing 0.1% Tween 20) for 5 min per wash before applying the goat anti-DNP primary antibody diluted at 1:10,000 in blocking buffer for 2 hours under agitation in the dark. The membrane was then washed 6 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 hours 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 each under agitation and was dried overnight before imaging the membrane.

Amino acid analysis (AAA)

Samples containing 25 μ M A β 40 and 5 μ M 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 μ L of 25 μ M peptide samples and performing a liquid phase hydrolysis using 200 μ L 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 μ L. The samples were analyzed on a cation-exchange chromatography column using a L-8800 Hitachi analyzer and a post column ninhydrin reaction detection system.

Electrospray ionization mass spectrometry (ESI-MS)

Both monomeric and fibrillar proteins were desalted first using the Amicon Centrifugal Filter (Millipore Sigma, Burlington, MA) before analyzed by ESI-MS. 250 μ L of peptide at 150 μ M 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 using PB buffer, the retentate was collected and volume adjusted to 250 μ L. The protein concentration was then determined using the Bradford protein concentration assay (Sigma-Aldrich, St. Louis, MO).

Desalted A β 40 monomer and fibril samples were analyzed by ESI-MS after 4 hours of irradiation in the absence and presence of MB or OPE1²⁻ (25 μ M protein with 1 μ M photosensitizer). Before analysis, A β 40 fibrils were digested using the Endoproteinase Lys C enzyme (New England BioLabs, Ipswich, MA) at a 1/50 (w/w) enzyme to protein ratio. The digestion was performed by incubating the samples at 37 °C for 16 hours. Digested A β 40 monomers and fibrils were diluted to 5 μ g/mL using acetonitrile with 1% TFA and were analyzed under continuous ESI-MS spray on SYNAPT G2 Mass Spectrometer. 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 for generation of calculated and comparison to observed mass ion packet.

Size exclusion chromatograph (SEC)

The unincubated and incubated A β 40 samples were analyzed by SEC to quantify the amounts of monomers present in the samples after 72 hours of incubation. Before injecting the sample on the HPLC column, 60 μ L of 50 μ M A β 40 was centrifuged for 15 min at 14,000 rpm to remove insoluble aggregates. Supernatant (50 μ L) was injected onto a BioSec-SEC-s2000 (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 an Agilent 1100 series HPLC (Agilent Technology, Santa Clara, CA). Absorbance at 215 nm was monitored. Background signal was subtracted using the Agilent ChemStation software and percentages of soluble monomers were calculated relative to the protein present in the unincubated samples.

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 OPE₁²⁻. After irradiation, the peptide solution 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 ellipticity⁸⁰.

TEM imaging

A β 40 samples were diluted to 5 μM using MilliQ water. After the grids were glow discharged (Harrick Plasma Cleaner, Carson City, NV) for 30 seconds, each sample was loaded onto a grid 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 a beam current of 8.0 μA and an accelerating voltage of 80 keV.

Cell toxicity assay

Neuroblastoma SHSY-5Y cells were cultivated in DMEM media containing 1% PS and 10% FBS at 37°C and 5% CO₂. When the 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 to 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.

All atom molecular dynamics (MD) simulation

The initial configurations for the A β fibril - OPE₁²⁻ simulations were built with UCSF Chimera⁸¹. The fibril structure, 2LMN⁶⁸ was obtained from the Protein Data Bank. In this work, we prepared an A β 9-40 protofibril made of 24 peptides to evaluate OPE₁²⁻ binding sites. 12 OPEs were positioned around the protofibril at a distance of 10 Å away from the protofibril surface. The anionic OPE₁²⁻ was built using the GaussView 5 package and geometry optimizations was carried out with Gaussian 09.⁸² Simulations were prepared using the AMBERTools suite⁸³. Parameters for simulating the protofibril 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^{84,85}. Each system was solvated in explicit water molecules using the TIP3P model and a total of 48 counter Na⁺ ions were added to neutralize the system. MD was performed using the AMBER MD package as previously described⁸⁶. Coordinates for the single A β 9-40 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.

OPE₁²⁻ binding sites on the protofibril surface were analyzed using UCSF 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.

ASSOCIATED CONTENT

Supporting Information: Reverse phase HPLC of A β 40 monomers before and after irradiation in the presence of MB and OPE₁²⁻; TEM images of A β 40 after incubation of 50 μ M monomers either alone (A) or in the presence of 2.6 μ M non-sonicated A β 40 fibrils (B) or 2.6 μ M sonicated A β 40 fibrils (C).

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A.M.F., D.O., F.A.M., T.D.M., J.S.M., D.G.E., D.G.W., and E.Y.C. designed research; A.M.F., D.O., F.A.M., J.H., F.M., T.D.M., G.B., and L.M.P. performed research; A.M.F., D.O., F.M., J.S.M., D.G.E. and E.Y.C. analyzed data; A.M.F. and E.Y.C. wrote the paper.

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ABBREVIATIONS

AAA: amino acid analysis; A β : amyloid β ; CD: circular dichroism; CTAB: cetyltrimethylammonium bromide; DMEM: Dulbecco's Modified Eagle's Medium; DMSO: dimethyl sulfoxide; DNPH: 2,4-Dinitrophenylhydrazine; ESI-MS: electrospray ionization mass spectrometry; FBS: fetal bovine serum; GAFF: generalized force field; HCl: hydrochloride acid; RP-HPLC: reverse phase high performance liquid chromatography; MB: methylene blue; MD: molecular dynamics; NaCl: sodium chloride; OPE: oligo-p-phenylene ethynylene; PDB: protein databank; PDT: photodynamic therapy; PS: penicillin-streptomycin; TPBS: pH 7.4 phosphate

buffered saline containing 0.1% Tween 20; TEM: transmission electron microscopy; TFA: trifluoroacetic acid

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Table of Content

Supplementary Information

Controlled and Selective Photo-oxidation of Amyloid- β Fibrils by Oligomeric *p*-Phenylene Ethynylenes

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Figure S1: Reverse phase HPLC chromatographs of soluble A β 40 before and after irradiation in the presence of MB or OPE $_1^{2-}$. Unincubated A β 40 monomers, whether non-irradiated or irradiated in the presence of OPE $_1^{2-}$, display a similar elution time that corresponds to 44.6-44.4% acetonitrile. When A β 40 monomers are irradiated in the presence of MB, the elution profile of A β 40 monomers significantly changed. The main peak eluted earlier (43.5% acetonitrile), which shows that A β 40 peptide became more hydrophilic, which is consistently with the oxidation of the

peptide. Also, the elution profile is broad with multiple peaks, indicating the presence of a several populations of A β 40. Experimental method: 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 OPE₁²⁻ or MB (5 μ M protein with 1 μ M photosensitizer). 110 μ L of 5 μ M protein was centrifuged at 14,000 rpm for 15 minutes. The supernatant (100 μ L) 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). A β 40 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.

Figure S2: TEM images of A β 40 after incubation of 50 μ M monomers either alone (A) or in the presence of 2.6 μ M non-sonicated A β 40 fibrils (B) or 2.6 μ M sonicated A β 40 fibrils (C). Only A β protofibrils produced by sonication promoted fast peptide fibrillation.