Pentameric Thiophene-Based Ligands that Spectrally Discriminate Amyloid-β and Tau Aggregates Display Distinct Solvatochromism and Viscosity-Induced Spectral Shifts

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Pentameric thiophene based ligands that spectrally discriminate amyloid-β and tau aggregates display distinct solvatochromism and viscosity induced spectral shifts.


Abstract: A wide range of neurodegenerative diseases are characterized by the deposition of multiple protein aggregates. Ligands for molecular characterization and discrimination of these pathological hallmarks are thus important for understanding their potential role in pathogenesis as well as for clinical diagnosis of the disease. In this regard, luminescent conjugated oligothiophenes (LCOs) have proven useful for spectral discrimination of amyloid-beta (Aβ) and tau neurofibrillary tangles (NFTs), two of the pathological hallmarks associated with Alzheimer’s disease. Herein, the solvatochromism of a library of anionic pentameric thiophene-based ligands, as well as their ability to spectrally discriminate Aβ and tau aggregates, were investigated. Overall, the results from this study identified distinct solvatochromic and viscosity-dependent behavior of thiophene-based ligands that can be applied as indices to direct the chemical design of improved LCOs for spectral separation of Aβ and tau aggregates in brain tissue sections. The results also suggest that the observed spectral transitions of the ligands are due to their ability to conform by induced fit to specific microenvironments within the binding interface of each particular protein aggregate. We foresee that these findings might aid in the chemical design of thiophene-based ligands that are increasingly selective for distinct disease-associated protein aggregates.

Keywords: Luminescent conjugated oligothiophenes • protein aggregates • fluorescence • solvatochromism • imaging

Introduction

Conjugated polymers have many unique photophysical properties which render them useful in a variety of applications within the fields of chemistry, molecular biology, and medicine. Luminescent conjugated polymers (LCPs) account for a growing number of developing sensors and probes as their unique properties make them useful reporters in the detection of ions, DNA, and proteins, to name just a few.[1-4] These sensors mainly employ the efficient light harvesting properties or the conformation-sensitive optical properties of the LCPs. The latter is particularly observed for LCPs with a repetitive flexible thiophene backbone, as conformational restriction of the thiophene rings leads to a distinct optical fingerprint.[5]

In recent studies, it has been shown that LCPs can function as target-specific chameleons that change color depending on the structural motif of the target molecule, even in complex samples such as tissue sections.[6-8] This intrinsic property of LCPs make them useful as selective probes for identifying and distinguishing protein deposits consisting mainly of fibrils with a repetitive cross-beta sheet structure. Accumulation of such proteinaceous deposits is the histopathological hallmark of several devastating diseases, including Alzheimer’s disease (AD) and prion diseases.[9,10] In addition, novel chemically defined thiophene scaffolds, denoted luminescent conjugated oligothiophenes (LCOs), have been utilized as specific ligands for a variety of disease associated protein aggregates, as well as for optical in vivo imaging of protein aggregates in real time.[11-16]

Anionic LCOs have proven particularly useful for spectral discrimination of amyloid beta (Aβ) deposits and tau neurofibrillary tangles (NFTs), the two major pathological hallmarks of AD.[11,12,17] Ligands with molecular scaffolds other than thiophene showing selectivity towards tau or Aβ deposits have also been presented and
these studies showed that minor chemical alterations of the molecular scaffold could influence the specificity toward either tau or Aβ aggregates.[18,19] Recently, aminonaphthalenyl 2-cyanoacrylate-based probes were also shown to fluoresce and discriminate between different types of protein deposits in brain.[20] The discriminating capability of these dyes was due to the stabilization of the ground versus excited states of these probes as a function of the polarity of the binding pocket on the amyloid. Hence, although most protein deposits share a repetitive cross-beta sheet structure, possible differences in the binding pocket microenvironments should be considered when designing ligands towards distinct protein aggregates.

In previous comparisons of structurally related LCOs, it was suggested that for optimal spectral separation of Aβ deposits and tau tangles, an LCO-based ligand should comprise a conformationally flexible backbone consisting of five to seven thiophene units and terminal carboxyl groups extending the conjugated thiophene backbone.[12,17] Upon binding to protein aggregates, LCOs also exhibit decreased Stokes shifts, red-shifted excitation maxima and blue-shifted emission maxima compared to free dyes in solution.[11,12,17] In an effort to elucidate the specific structural features that contribute to enhanced spectral separation between protein aggregates and the photophysical behavior of LCOs bound to protein deposits in more detail, we herein investigated solvatochromism and the effects of solvent viscosity on a group of structurally similar, pentamer, anionic oligothiophene probes. LCOs that displayed spectral variations for Aβ deposits and NFTs also showed distinct solvatochromism and decreased Stokes shifts due to increased solvent viscosity. Hence, these photophysical assessments might aid in the design of LCOs for sensitive optical discrimination of Aβ and tau deposits.

Results and Discussion

Solvatochromism of a library of anionic LCOs: Solvatochromic behavior of small amyloid ligands can be used to approximate the amyloid fibril binding site polarity, as well as the relative change in the dipole moment for individual ligands.[20-23] Therefore we tested the solvatochromic behavior of a subset of previously reported anionic pentamer LCOs (Figure 1).[11,12,17] These LCOs were chosen as their molecular composition varies in anionic substitution patterns and in backbone rigidity, as well as in terminal functional groups extending the thiophene backbone. To assess the solvent sensitivity of these LCOs, Lippert-Mataga plots (Stokes shift vs orientation polarizability) were used.[24] The relative slopes of the fitted lines allows for a comparison of the solvent sensitivity of each of the LCOs tested by means of the orientation polarizability, Δf, for each solvent, determined by the equation,

\[ \Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} \frac{n^2 - 1}{2n^2 + 1} \]

in which ε is the dielectric constant and n the refractive index of each solvent. The calculated Δfs are shown in Table S1 (Supporting information). The Stokes shifts, Δλ, were given by,

\[ \Delta \lambda = \lambda_{\text{EX}} - \lambda_{\text{EM}} \]

where \( \lambda_{\text{EX}} \) and \( \lambda_{\text{EM}} \) are the wavelengths corresponding to the excitation- or the emission maximum, respectively. The results from the solvatochromism study are shown in Figure 1 and Table 1. p-FTAA and HS-72 displayed the highest slope values as these dyes have the highest degree of conformational freedom along the backbone of the thiophene backbone. When the central thiophene ring was replaced with a selenophene (p-FTAA-Se), the relative slope was decreased and still further reduced through the introduction of a central phenyl (p-FTAA-Ph). A decrease in the relative slopes of the fitted lines was also observed when replacing the terminal carboxyl groups with hydrogen (p-HTAA). The combination of chromophore planarization and polarization occurs in natural processes, such as the chemistry of vision,[25,26] and earlier studies[27,28] have also shown that similar phenomena occurs in tetrameric oligothiophenes. Thus, conformational restrictions of the LCO backbone will most likely influence the polarization of the dye, since chromophore planarization and polarization are coupled processes. Likewise, substituting the polarizable carboxyl groups with hydrogens will influence the polarization of the molecule and the solvent sensitivity. Furthermore, the carboxyl groups can also acts as π-acceptors. A previous study,[17] comparing p-FTAA, HS-72, p-FTAA-Se and p-FTAA-Ph, has shown that p-FTAA and HS-72 are efficient for spectral discrimination of Aβ deposits and NFTs, whereas p-FTAA-Se was less effective, and p-FTAA-Ph completely lacked the ability to distinguish the two aggregated species. In addition, carboxyl groups extending the conjugated thiophene backbone have been shown to be an additional molecular determinant for achieving optimal spectral separation of Aβ deposits and NFTs.[12] Overall, the trend in solvatochromic behavior of the LCOs supports a correlation between the solvent sensitivity of the LCOs and their ability for spectral separation of Aβ deposits and tau tangles.

<table>
<thead>
<tr>
<th>LCO</th>
<th>Slope: solvent</th>
<th>Slope: viscosity</th>
<th>Stokes shift ethyl acetate</th>
<th>Stokes shift glycerol</th>
<th>Stokes shift Aβ 1-42 fibrils</th>
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</thead>
<tbody>
<tr>
<td>HS-72</td>
<td>208.7</td>
<td>30.0</td>
<td>95</td>
<td>83</td>
<td>51</td>
</tr>
<tr>
<td>p-FTAA</td>
<td>205.7</td>
<td>23.0</td>
<td>114</td>
<td>103</td>
<td>48</td>
</tr>
<tr>
<td>p-FTAA-Se</td>
<td>148.3</td>
<td>10.0</td>
<td>107</td>
<td>82</td>
<td>54</td>
</tr>
<tr>
<td>p-HTAA</td>
<td>69.0</td>
<td>23.3</td>
<td>125</td>
<td>106</td>
<td>46</td>
</tr>
<tr>
<td>p-FTAA-Ph</td>
<td>30.7</td>
<td>6.03</td>
<td>116</td>
<td>110</td>
<td>116</td>
</tr>
</tbody>
</table>

As solvatochromism Stokes shifts can provide insight regarding protein binding site polarity,[19,21] we next compared the Stokes shifts obtained from the solvatochromism experiments with the Stokes shifts from the LCOs bound to recombinant Aβ1-42 fibrils (Table 1). All the LCOs, except for p-FTAA-Ph, displayed considerably reduced Stokes shifts for Aβ1-42 fibrils compared to ethyl acetate, indicating that the Aβ1-42 binding pocket is substantially more non-polar than ethyl acetate (ε = 6.08). In a recent study,[20] using aminonaphthalene 2-cyanoacrylate dyes, the dielectric constants of the binding pocket of Aβ deposits in tissue was determined to be roughly similar to diethyl ether (ε = 4.27) and in a similar study[23] using Nile Red, the Aβ1-42 fibrils binding site polarity was predicted to have a dielectric constant lower than 8.
Figure 1. Chemical structures (first column), Lippert-Mataga solvatochromism plots (second column) and viscosity plots (third column) of the pentameric LCOs, A) HS-72, B) p-FTAA, C) p-FTAA-Se, D) p-HTAA and E) p-FTAA-Ph. For the solvatochromism, solvents of increasing polarity in the following order: ethyl acetate, octanol, dimethyl sulfoxide (DMSO), ethylene glycol, methanol, and water were used. For the viscosity experiments, LCOs were mixed in solutions of ethylene glycol and glycerol with increasing concentrations of glycerol. The LCO concentration was 300 nM for all experiments.
Previous studies indicate that molecules exhibiting abnormally low Stokes shift are most likely undergoing secondary effects, such as hydrogen bonding or binding induced conformational restrictions. Thus, the low Stokes shifts observed for the LCOs bound to recombinant Aβ1–42 fibrils might also be due to such secondary effects. In fact, p-FTAA-Ph displayed a similar Stokes shift for Aβ1–42 fibrils as the solvents and this dye has less conformational freedom than the other LCOs so the dye is most likely prevented from undergoing additional conformational restriction upon binding to the fibrils. Furthermore, in contrast to the solvent shifts, Aβ1–42 binding also caused a significant bathochromic shift in the excitation spectra for all the LCOs except p-FTAA-Ph, indicating that a planarization of the ground state of the probes occurs upon interaction with the fibrils (SI, Figure S1).

**Viscosity dependent excitation- and emission profiles of anionic LCOs:** As the considerably reduced Stokes shifts observed for LCOs bound to Aβ1–42 fibrils could be due to conformational restrictions within the binding pocket, we proceeded to investigate the role of such restrictions on Stokes shift upon fibril binding using a solvent viscosity model. Solvent viscosity can be used to assess probe behavior based on conformational restrictions, since highly viscous solvents exhibit slow solvent reorientation, restrict conformational freedom, and reduce vibrational modes of relaxation, resulting in emission before complete solvent reorientation and a hypsochromic shift of the probe’s emission spectra. In some cases, increasing viscosity can also stabilize the ground state, causing a bathochromic shift in the excitation spectra and further reducing the Stokes shift. By using two solvents of similar polarity, (ethylene glycol and glycerol), but with differing viscosities, we were able to simulate the conformational restrictions with minimal changes in polarity. For p-FTAA and HS-72, increasing the ratio of glycerol to ethylene glycol resulted in a red-shift of the excitation spectrum and blue-shift of the emission spectrum, (Figure 2). Hence, both of these dyes displayed a viscosity-dependent decrease in their Stokes shifts and considerable Stokes-viscosity slopes, when plotting the Stokes shifts versus percent of glycerol (Figure 1, Table 1). p-HTAA also displayed a substantial Stokes-viscosity slope, although the blue-shift in the emission spectrum was less pronounced. In contrast, p-FTAA-Se and p-FTAA-Ph, displayed minor Stokes-viscosity slopes. As mentioned above, p-FTAA-Ph with a central phenyl ring has restricted degrees of conformational freedom and for p-FTAA-Se, replacement of the sulfur atom with selenium in the central ring causes the central portion of the backbone to be more planar and less flexible. Compared to the solvatochromism experiments, the viscosity measurements better reflect Stokes shift changes upon conformational restriction of the molecule. Here it becomes more apparent that p-FTAA-Ph and p-FTAA-Se, displayed reduced chromic responsiveness due to inherent conformational restrictions of the conjugated backbone.

Although the effort of increasing the conformational restrictions via viscosity reduced the Stokes shift, the spectral changes that occur upon binding to Aβ1–42 fibrils cannot be reproduced by solvent-only restrictions (Figure 2, Table 1). The occurrence of vibronic peaks, the bathochromic excitation shift, and the hypsochromic emission shift all indicate that the LCOs are much more constrained in the ground state upon binding to Aβ1–42 fibrils, even when compared to the most viscous condition of 100% glycerol. These signature peaks in both the excitation and emission spectra when bound to Aβ1–42 fibrils can be observed for all of the probes with the exception of p-FTAA-Ph. In addition, similar to the fluorescent amyloid ligand thioflavin T (ThT), all the LCOs displayed an enhanced fluorescence upon binding to amyloid fibrils (SI, Figure S2). Recent studies indicate that the binding of ThT to amyloid fibrils are highly dependent on interactions with the aromatic and hydrophobic sides chains of the protein fibrils.
Thus, ThT fluorescence is highly sensitive to local interactions occurring when the dye is bound to amyloid fibrils. In addition, it was recently shown that efficient binding of the most conventionally used amyloid ligand, Congo Red, to amyloid fibrils is highly dependent on electrostatic interactions and hydrogen bonding.\cite{32}

Most likely such interactions are also relevant for the conformational restriction of the anionic LCOs upon binding to Aβ1-42 fibrils and the interplay of these interactions cannot be completely mimicked by the solvent-only models presented above. However, the solvatochromism and viscosity indices did correlate with the pentameric LCOs optical ability to distinguish Aβ and tau deposits. As reported earlier,\cite{12,17} LCOs having carboxyl groups extending the pentameric thiophene backbone, as well as having greater conformational freedom, tend to perform better as probes for the detection of conformational differences between protein aggregates. As shown herein, such LCOs also displayed distinct solvatochromism and decreased Stokes shifts due to increased solvent viscosity. These fundamental photophysical assessments might thus be utilized to predict the LCOs ability to act as sensitive optical discriminators of Aβ and tau deposits.

**Synthesis and evaluation of three additional LCOs:** In order to test our hypothesis that pentameric LCOs for optimal spectral discrimination of Aβ deposits and NFTs should display distinct solvatochromism as well as spectral shifts due to solvent viscosity, three novel anionic pentameric LCO analogues to p-FTAA were synthesized (Figure 3). Firstly, the terminal carboxyl groups were replaced by ketones, resulting in p-KTAA, an anionic pentamer with the same central trimer building block as p-FTAA and neutral polarizable π-acceptor groups (ketones) extending the thiophene backbone instead of negatively charged carboxyl groups. Secondly, the positions of the acetic acid side chains were altered on the trimer building block to render HS-84, an isomer to p-FTAA having the acetic side chains of the trimeric building block tail-to-tail instead of head-to-head. Thirdly, a pentamer (HS-42) lacking the terminal carboxyl groups extending the conjugated backbone, but displaying the same amount of net charge (-4) as p-FTAA was synthesized.

The new LCOs were synthesized in a similar fashion as previously reported LCOs.\cite{11,12,17} Thiophene trimer \textsuperscript{13} was used as precursor for the synthesis of target compounds p-KTAA and
HS-42 (Scheme 1). Electrophilic aromatic substitution on trimer 1 using N-bromosuccinimide in DMF gave dibrominated thiophene trimer 2 in 94% yield. Compound 2 was subjected to a Suzuki coupling with 5-acetyl-2-thienylboronic acid (3) using K$_2$CO$_3$ and the palladium-catalyst PEPPSI™-IPr. Due to solubility problems, after workup, the crude methylester pentamer was subsequently hydrolyzed with 1 M aqueous NaOH in dioxane and water to give p-KTAA in an overall yield of 83 % over two steps. Compound 4[17] was coupled to compound 2 according to the above-mentioned Suzuki conditions affording pentamer 5 in 48 % yield. Hydrolysis with 1 M aqueous NaOH in dioxane and water gave HS-42 quantitatively (Scheme 1). The synthetic approach towards pentameric oligothiophene HS-84 required the dimeric thiophene 8 (Scheme 2). This intermediate was prepared according to the same palladium cross-coupling conditions as described above using monomers 6[33] and 7, followed by esterification under acidic conditions using methanol as solvent and nuclease in an overall yield of 71 % over two steps. Bromination of the intermediate 8 with N-bromosuccinimide in DMF afforded the key precursor 9 in 73 % yield. Following the previous procedure dibrominated dimer 9 was coupled to 2,5-thiophenediylbisboronic acid (10) yielding methyl ester pentamer 11 in 80 %. Final hydrolysis as for HS-42 and p-KTAA gave HS-84 quantitatively (Scheme 2). After synthesis and purification, the solvatochromism and viscosity-dependent spectral changes of the dyes were assessed as described above.

| Table 2. Slope values of Lippert-Mataga plots for additional pentamers. |
|-----------------------------|-----------------|-----------------|
| LCO     | Slope: solvent | Slope: viscosity |
| p-KTAA  | 774.6          | 39.8            |
| HS-84   | 166.1          | 1.4             |
| HS-42   | 1.85           | 30.0            |

The solvatochromic Lippert-Mataga plot for the three novel anionic pentameric LCOs are shown in figure 3 and the slope values for the respective LCOs are summarized in Table 2. Similar to p-HTAA, HS-42 also displayed low solvent sensitivity due to the absence of terminal carboxyl groups extending the conjugated thiophene backbone. HS-84 showed a similar solvent sensitivity as p-FTAA, verifying that LCOs having terminal carboxyl groups reveal high solvent sensitivity. In addition, p-KTAA displayed an even higher slope value than p-FTAA, verifying that elongation of the conjugated thiophene backbone with polarizable π-acceptor groups other than carboxyl groups, are possible for achieving LCOs displaying high solvent sensibility. In the viscosity plot, HS-42 and p-KTAA showed high slope values, whereas HS-84 lacked the viscosity-induced changes of the Stokes shift (Figure 3, Table 2).
Hence, it appears that HS-84 has less conformational freedom along the backbone than p-FTAA, indicating that changing the position of the acetic acid side chains of the trimeric building block from head-to-head to tail-to-tail might induce alternative intramolecular interactions, such as hydrogen bonding or sulphur-oxygen interactions,\(^{[34]}\) resulting in backbone rigidity. In addition, it appears that the positioning of acetic acids moieties on adjacent thiophene rings, as in HS-42, induces an increase in steric hindrance between pendant groups without hindering the flexibility of the conjugated backbone. For all the newly synthesized LCOs, the spectral changes that occur upon binding to Aβ1-42 fibrils could not be reproduced by solvent-only restrictions (SI, Figure S3 and S4). Furthermore, all the three LCOs displayed an enhanced fluorescence upon binding to amyloid fibrils (SI, Figure S5). Overall, the results from the solvent experiments predict p-KTAA to be an efficient LCO for spectral discrimination between Aβ and NFTs, while HS-42 and HS-84 should display similar emission profiles for these aggregated species.

To verify the three new pentamers’ abilities to distinguish between Aβ and tau deposits, the dyes were utilized for staining of human brain tissue with AD pathology (Figure 4). Indeed, p-KTAA, with the highest solvent sensitivity and the most dynamic range of conformational freedom based on viscosity slopes, proved to be most efficient in terms of ability to spectrally distinguish between immunopositive Aβ deposits and NFTs. p-KTAA bound to Aβ-deposits showed an emission maximum around 570 nm, whereas the p-KTAA spectrum from NFTs was red-shifted, having an emission maximum around 595 nm (Figure 4A). Both HS-84 and HS-42 displayed similar emission spectra for the two aggregated entities, verifying that LCOs that are efficient for spectral separation of Aβ deposits and NFTs need to display solvent sensitivity as well as viscosity induced Stokes shifts. Therefore, the results indicate that the indices of solvent sensitivity (solvatochromism) and conformational freedom (viscosity) can be utilized as predictive determinants for achieving superior LCOs for spectral separation of differing protein aggregates.

**Conclusion**

Herein, we show that LCOs that are able to spectrally distinguish Aβ-deposits and NFTs display distinct solvatochromism as well as viscosity-dependent optical transitions. In addition, the spectral transitions that arise from the LCOs interactions with specific protein aggregates are most likely due to differences in binding pocket polarities and the conformational restrictions of the respective protein aggregates. Overall, we have demonstrated that a combination of basic photophysical assessments can facilitate the chemical design of novel thiophene-based ligands that can distinguish different protein aggregate topologies. The results presented also underline that the microenvironment in the binding pockets of distinct protein aggregates differ and this should be considered when designing protein aggregate specific ligands.

**Experimental Section**

Full experimental details including additional characterization data and NMR spectra of new compounds are given in the Supporting Information.

**Acknowledgements**

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**The coaxed chameleon.**

*Rozalyn Simon, Dr. Hamid Shirani, Dr. K. O. Andreas Åslund, Dr. Marcus Bäck, Prof. V. Haroutunian, Prof. Sam Gandy and Dr. K. Peter R. Nilsson*  
................ Page – Page

**Pentameric thiophene based ligands that spectrally discriminate amyloid-β and tau aggregates display distinct solvatochromism and viscosity induced spectral shifts.**

Distinct solvatochromic and viscosity dependent optical behaviour were observed for thiophene-based optical ligands that can be utilized for spectral assignment of disease-associated protein aggregates. The observed spectral transitions of the ligands are most likely due to their ability to conform by induced fit to specific microenvironments within the binding interface of each particular protein aggregate.
Supporting Information

Supporting information Table S1. Calculated orientation polarizability, $\Delta f$, for each solvent.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\Delta f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.32</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.31</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>0.27</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.26</td>
</tr>
<tr>
<td>Octanol</td>
<td>0.23</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.19</td>
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</table>

**Supporting information figure S1:** Normalized excitation and emission spectra of 300 nM HS-72, p-FTAA, p-FTAA-Se, p-HTAA and p-FTAA-Ph in different solvents. As a comparison of excitation and emission spectra for the LCOs bound to Aβ1-42 fibrils (10 µM) in PBS are shown (red).
Supporting information figure S2: Emission spectra of 300 nM HS-72, p-FTAA, p-FTAA-Se, p-HTAA and p-FTAA-Ph in PBS (black) or bound to recombinant Aβ1-42 fibrils (10 µM) (red).

Supporting information figure S3: Normalized excitation and emission spectra of 300 nM p-KTAA, HS-84 and HS-42 in different solvents. As a comparison of excitation and emission spectra for the LCOs bound to Aβ1-42 fibrils (10 µM) in PBS are shown (red).
Supporting information figure S4: Normalized excitation and emission spectra of 300 nM p-KTAA, HS-84 and HS-42 in 100 % ethylene glycol (grey) 50 % ethylene glycol and 50 % glycerol (yellow), or 100 % glycerol (red). As a comparison of excitation and emission spectra for the LCOs bound to Aβ1-42 fibrils (10 µM) in PBS are shown (black).

Supporting information figure S5: Emission spectra of 300 nM p-KTAA, HS-84 and HS-42 in PBS (black) or bound to recombinant Aβ1-42 fibrils (10 µM) (red).
EXPERIMENTAL DETAILS

LCO synthesis. HS-72, p-FTAA, p-HTAA, p-FTAA-Se and p-FTAA-Ph were synthesized as described previously. \(^1\)\(^2\). p-KTAA, HS-42 and HS-84 were synthesized as described in the following sections.

General methods: NMR spectra were recorded on a Varian 300 instrument (Varian Inc., Santa Clara, CA, USA) operating at 300 MHz for \(^1\)H and 75.4 MHz for \(^{13}\)C, using the residual solvent signal as reference. IR spectra were acquired on a Perkin-Elmer Spectrum 1000 using KBr pellets. Chemicals and solvents were obtained from commercial sources and used as received. TLC was carried out on Merck precoated 60 F254 plates using UV-light (\(\lambda = 254 \text{ nm and 366 nm}\)) and charring with ethanol/sulfuric acid/p-anisaldehyde/acetic acid 90:3:2:1 for visualization. Column chromatography was carried out on silica gel Merck 60 (40–63 µm). MALDI-TOF-spectra were recorded on a Voyager-DE STR Biospectrometry Workstation using \(\alpha\)-cyano-4-hydroxycinnamic acid as a matrix and reference.

Compound 2: Thiophene trimer 1 (1.00 g, 2.55 mmol) was dissolved in DMF (10 mL) and the solution was cooled to 0 °C. NBS (0.681 g, 3.83 mmol) was added portion wise during one minute. The solution was allowed to attain room temperature during 2 h, again cooled to 0 °C, more NBS (0.226 g, 1.27 mmol) was added portion wise during one minute, and the solution was allowed to attain room temperature. Dilution with EtOAc, washing with brine, drying, filtering, concentration, and FC (toluene, toluene/EtOAc 18:1) gave 2 (1.32 g, 94%) as a slightly yellow solid. mp 81-83 °C IR (neat) 1733, 1503, 1432, 1417, 1341, 1310, 1203, 1178, 987, 895, 843, 783 cm\(^{-1}\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.09 (s, 2H), 7.03 (s, 2H), 3.73 (s, 6H), 3.71 (s, 4H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 170.9, 135.0, 134.2, 134.2, 133.1, 127.9, 111.9, 52.5, 34.6. MALDI-TOF: \(m/z\) calcld for C\(_{18}\)H\(_{15}\)Br\(_2\)O\(_3\)S\(_3\) (M+H): 548.8. Found: 548.8.
**p-KTAA:** To a mixture of dibrominated thiophene trimer 2 (0.120 g, 0.219 mmol), 5-Acetyl-2-thienylboronic acid (3) (0.111 g, 0.653 mmol) and K$_2$CO$_3$ (0.182 g, 1.31 mmol) in degassed 1,4-dioxane/methanol (8 : 2, 5 mL) was added PEPPSI-iPr$^\text{™}$ (0.0075 g, 0.011 mmol). The mixture was heated to 70 °C for 20 min, cooled to rt, adjusted to pH 4 by 1M HCl, extracted with DCM (3×30 mL/mmol), and washed with water (3×30 mL/mmol) and brine (30 mL). During workup the methyl ester protected product precipitated and was washed thoroughly with warm dioxane and added to a solution of dioxane/NaOH (1M, aq.) and heated at 75 °C until everything dissolved. Acidification by HCl (1 M. aq.) resulted in precipitation of the deprotected neutral product, which was washed with water and dried (0.111 g, 83%) and subsequently dissolved in aqueous NaOH (1.5 equivalents per carboxylic acid) to give p-KTAA as a red sodium salt. mp >300 °C. IR (neat) 1645, 1583, 1447, 1384, 1312, 1282, 1078, 1033, 934, 799 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 7.85 (d, $J = 4.0$ Hz, 2H), 7.46 (s, 2H), 7.40 (d, $J = 4.0$ Hz, 2H), 7.30 (s, 2H), 3.75 (s, 4H), 2.49 (s, 6H). $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 190.3, 171.4, 143.4, 142.4, 135.1, 134.6, 133.4, 133.3, 132.3, 130.3, 127.7, 125.3, 34.7, 26.3. MALDI-TOF: $m/z$ calcd for C$_{28}$H$_{21}$O$_6$S$_5$ (M+H)$^+$: 613.0. Found: 613.1.

**HS-42-ester (5):** The compound was prepared according to the same Suzuki coupling procedure as employed for the synthesis of p-KTAA using trimer 2 (0.1 g, 0.182 mmol), compound 4 (0.1 g, 363 mmol), K$_2$CO$_3$ (0.15 g, 1.1 mmol), and PEPPSI-iPr$^\text{™}$ (5 mol %) in degassed 1,4-dioxane/methanol (8 : 2, 5 mL). The reaction mixture was extracted with DCM (3×30 mL), washed with water (2×30 mL), brine (50 mL) and the combined organic phases were dried over MgSO$_4$ and the solvent was evaporated under reduced pressure. The crude product, red oil, was subjected to column chromatography [heptane/EtOAc (4:1→1:1)], affording pentamer 5 (0.61 g, 48 %) as red oil. IR (neat) 1734, 1435, 1332, 1263, 1197, 1171, 1015, 832, 711 cm$^{-1}$. $^1$H NMR
(300 MHz, CDCl₃) δ 7.25 (d, J = 5.2 Hz, 2H), 7.18 (s, 2H), 7.13 (s, 2H), 7.05 (d, J = 5.2 Hz, 2H), 3.81 (s, 4H), 3.80 (s, 4H), 3.75 (s, 6H), 3.74 (s, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 171.4, 171.2, 135.5, 134.3, 133.5, 132.8, 131.1, 130.7, 130.5, 130.0, 127.7, 125.0, 52.4, 52.4, 35.0, 34.8. MALDI-TOF: m/z calcld for C₃₂H₂₉O₈S₅ (M+H)⁺: 701.0. Found: 701.2.

**HS-42-salt:** NaOH (1 M, 1.5 equiv./ester) was added to a solution of ⁵ (0.06 g, 0.087 mmol) in 1,4-dioxane (5 mL) and heated to 60 °C for 16 h. Upon precipitation H₂O was added and the solution was lyophilized. The HS-42 salt was obtained quantitatively as a red solid. mp >300 °C. IR (neat) 1569, 1436, 1394, 1281, 918, 880, 835, 788, 752 cm⁻¹. ¹H NMR (300 MHz, D₂O) δ 7.40 (d, J = 5.2 Hz, 2H), 7.25 (s, 2H), 7.10 (s, 2H), 7.05 (d, J = 5.2 Hz, 2H), 3.76 (s, 4H), 3.74 (s, 4H). ¹³C NMR (75 MHz, D₂O) δ 179.80, 179.40, 135.2, 134.3, 133.8, 133.7, 131.60, 131.5, 131.3, 129.8, 126.5, 124.5, 37.9, 37.8. MALDI-TOF: m/z calcld for C₂₈H₂₁O₈S₅ (M+H)⁺: 645.0. Found: 645.2.

**Methyl 3’-(2-methoxy-2-oxoethyl)-[2,2’-bithiophene]-5-carboxylate (8):** The compound was prepared according to the same Suzuki coupling procedure as employed for the synthesis of p-KTAA using ⁶ (1 g, 4.25 mmol), ⁷ (0.73 g, 4.25 mmol) and K₂CO₃ (3 g, 22 mmol) in 1,4-dioxane/methanol (8:2, 40 mL). The reaction mixture was extracted with EtOAc (3×50 mL), washed with water (3×50 mL), brine (50 mL) and the combined organic phases were dried over MgSO₄ and the solvent was evaporated under reduced pressure. The crude product, off white solid, was dissolved in MeOH (50 mL), conc. sulfuric acid (1.0 mmol) was added and the mixture was heated at 70°C for 16 h. The mixture was neutralized with aq. Na₂CO₃ and the aqueous layer was extracted with DCM (3×30 mL). The combined organic phases were washed with water (3×30 mL), brine (30 mL) and dry with MgSO₄. The solvent was removed under reduced pressure and the residue was subjected to column chromatography [heptane/EtOAc (8:1)], yielding the dimer ⁸ (0.9 g, 71 %) as white solid. mp 66-67 °C. IR (neat) 1732, 1703, 1463, 1435, 1418, 1301, 1241, 1232, 1192, 1137, 1109, 1049, 1018, 912, 830 cm⁻¹. ¹H NMR
300 MHz, CDCl$_3$) $\delta$ 7.75 (d, $J = 3.9$ Hz, 1H), 7.29 (d, $J = 5.2$ Hz, 1H), 7.16 (d, $J = 3.9$ Hz, 1H), 7.06 (d, $J = 5.2$ Hz, 1H), 3.89 (s, 3H), 3.78 (s, 2H), 3.72 (s, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 171.1, 162.5, 162.5, 142.2, 134.1, 133.3, 132.5, 131.6, 130.8, 127.2, 125.8, 52.4, 52.4, 34.8.

MALDI-TOF: $m/z$ calcd for C$_{13}$H$_{13}$O$_4$S$_2$ (M+H)$^+$: 297.0. Found: 297.1.

**Methyl 5'-bromo-3'-(2-methoxy-2-oxoethyl)-[2,2'-bithiophene]-5-carboxylate (9):** The dimer 8 (0.787 g, 2.6 mmol) was dissolved in DMF (20 mL) and NBS (12.5 g, 70.4 mmol) in DMF (10 mL) was added. The reaction mixture was stirred for 16 h, water (50 mL) was added and extracted with DCM (3×50 mL), was washed with water (3×50 mL), brine (50 mL) and the combined organic phases were dried over MgSO$_4$. The solvent was removed under reduced pressure and the crude product yellow oil was subjected to column chromatography [heptane/EtOAc (8:1)], affording product 8 (0.72 g, 73 %) as light yellow solid. mp 93-94.5 °C. IR (neat) 1739, 1700, 1515, 1452, 1377, 1330, 1314, 1291, 1212, 1193, 1168, 1108, 1053, 989, 822, 813 cm$^{-1}$. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.73 (d, $J = 3.9$ Hz, 1H), 7.12 (d, $J = 3.9$ Hz, 1H), 7.04 (s, 1H), 3.89 (s, 3H), 3.73 (s, 3H), 3.71 (s, 2H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.7, 162.4, 140.7, 134.1, 133.9, 133.8, 133.3, 132.1, 127.6, 112.9, 52.5, 52.5, 34.6. MALDI-TOF: $m/z$ calcd for C$_{13}$H$_{13}$BrO$_4$S$_2$ (M+H)$^+$: 374.9. Found: 375.0.

**HS-84-ester (11):** The compound was prepared according to the same Suzuki coupling procedure as employed for the synthesis of p-KTAA using 9 (0.218 g, 0.582 mmol), 10 (0.050 g, 0.291 mmol), K$_2$CO$_3$ (0.200 g, 1.5 mmol), and PEPPSI-iPr™ (5 mol %) in degassed 1,4-dioxane/methanol (8 : 2, 15 mL). The reaction mixture was extracted with DCM (3×40 mL), washed with water (2×40 mL), brine (40 mL) dried over MgSO$_4$ and the solvent was evaporated under reduced pressure to give crude product as red solid. This was treated with warm EtOAc and filtrated to give the pure product 11 (0.156 g, 80%) as red solid. mp 184-185 °C. IR (neat)
1732, 1711, 1453, 1339, 1291, 1266, 1190, 1099, 785, 745 cm\(^{-1}\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.76 (d, \(J = 4.0\) Hz, 2H), 7.20 (d, \(J = 4.0\) Hz, 2H), 7.14 (s, 2H), 7.11 (s, 2H), 3.91 (s, 6H), 3.79 (s, 4H), 3.76 (s, 6H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 170.7, 162.3, 141.5, 136.6, 135.8, 134.0, 133.2, 132.1, 131.4, 127.1, 126.9, 125.1, 52.4, 52.3, 34.8. MALDI-TOF: \(m/z\) calcd for C\(_{30}\)H\(_{25}\)O\(_8\)S\(_5\) (M+H\(^+\)):\(673.0\). Found: 673.1.

**HS-84-salt:** Same procedure as descried for HS-42 by adding NaOH (1 M, 1.5 equiv./ester) to a solution of 10 (0.100 g, 0.150 mmol) in 1,4-dioxane (5 mL). The HS-84 salt was obtained quantitatively as a red solid. IR (neat) IR (neat) 1572, 1453, 1391, 1379, 1319, 1255, 984, 881, 826, 801, 784, 772 cm\(^{-1}\). \(^1\)H NMR (300 MHz, D\(_2\)O) \(\delta\) 7.43 (d, \(J = 3.9\) Hz, 2H), 7.00 (s, 2H), 6.99 (s, 2H), 6.97 (d, \(J = 3.9\) Hz, 2H), 3.63 (s, 4H). \(^{13}\)C NMR (75 MHz, D\(_2\)O) \(\delta\) 179.1, 169.5, 139.9, 139.7, 135.4, 135.0, 134.7, 131.3, 130.9, 128.1, 125.7, 124.8, 38.2. MALDI-TOF: \(m/z\) calcd for C\(_{26}\)H\(_{17}\)O\(_8\)S\(_5\) (M+H\(^+\)):\(617.0\). Found: 617.0.

**Solvent studies.** All probes were taken as anionic salts, dissolved directly into DMSO at 1.5 mM and from the DMSO stock, diluted to 300 nM in solvent of choice, and purged under nitrogen gas before fluorescence readings. Excitation- and emission spectra were recorded using a Tecan Saphire 2 fluorescence plate reader at room temperature.

**A\(\beta\) aggregate fluorescence studies.** A\(\beta\) aggregates were prepared using A\(\beta\)1-42 peptides purchased from R-peptide by dissolving them in 2 mM NaOH and diluting with PBS at pH 7.4 to a final concentration of 10 \(\mu\)M. Fibrils were formed by incubation at 37 °C for 24 hours. The kinetics of amyloid fibril formation was monitored by thioflavin T (ThT) and p-FTAA fluorescence until fibrils reached maturity. Probes were then added to 100 \(\mu\)L of the mature A\(\beta\) fibrils for a final probe concentration of 300 nM. The excitation and emission were recorded using a Tecan Saphire 2 fluorescence plate reader at room temperature.
**LCO staining of tissues and spectral analysis.** Frozen brain tissues from clinically and neuropathologically well-characterized cases of AD were obtained from the Alzheimer’s Disease Research Center, Mount Sinai School of Medicine, New York NY 10029, USA and informed consent for brain donation was obtained from the next-of-kin. The cryosections were brought to room temperature in a humidity chamber, fixed in ethanol, rinsed in phosphate buffer saline (PBS) pH 7.4 and then stained with each of the LCOs (300 nM in PBS) for 30 min and then rinsed with PBS and mounted with DAKO fluorescent mounting media. The mounting medium was allowed to solidify over night before the rims were sealed with nail polish and the staining result was analyzed using an inverted LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany), equipped with a 32 channel QUASAR GaAsP spectral array detector. Fluorescence emission spectra were collected using single wavelength excitation at 488 nm (Argon Laser) and emission spectra were collected between 488 - 687 nm. Fifteen regions of interest were sampled from either Aβ plaques or tau neurofibrillary tangles for each probe and the average was taken using Graphpad Prism software (GraphPad, La Jolla, CA, USA).

**REFERENCES**

