Substituent, Charge, and Size Effects on the Fluorogenic Performance of Amyloid Ligands: A Small-Library Screening Study

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ABSTRACT: Developing new molecular ligands for the direct detection and tracking of amyloid protein aggregates is key to understanding and defeating myriad neurodegenerative and other disorders including Alzheimer’s and Parkinson’s diseases. A crucial factor in the performance of an amyloid dye is its ability to detect the amyloid structural motif independent of the sequence of the amyloid-forming protomer. The current study investigates structure–function relationships of a class of novel phenyleneethynylene (PPE)-based dyes and fluorescent polymers using amyloid fibrils formed by two model proteins: lysozyme and insulin. A small library of 18 PPE compounds that vary in molecular weights, charge densities, water solubilities, and types and geometries of functional groups was tested. One compound, the small anionic oligo(phenylene ethylene) electrolyte OPE1, was identified as a selective sensor for the amyloid conformation of both lysozyme and insulin. On the basis of protein binding and photophysical changes observed in the dye from this set of PPE compounds, keys to the selective detection of the amyloid protein conformation include moderate size, negative charge, and substituents that provide high microenvironment sensitivity to the fluorescence yield. These principles can serve as a guide for the further refinement of the effective amyloid-sensing molecules.

INTRODUCTION

The function of a protein in living cells depends on the folding and stabilization of a native structure or set of conformers. Misassembly of proteins into β-sheet-enriched fibrillar aggregates is a hallmark of protein misfolding disorders such as, among many others, Alzheimer’s disease (AD) and Parkinson’s disease. Amyloid protein aggregation is a complex and heterogeneous process that begins with a native or native-like structure and ends with highly ordered fibrils that accumulate in the affected tissues. In AD, protein aggregation is believed to initiate a cascade of events culminating in neurodegeneration and cognitive decline decades before the onset of clinical symptoms, thereby protein aggregates are thus ideal biomarkers for early disease detection and therapeutic intervention.

Many studies have been launched to develop probes for detecting the aggregated conformation of disease-associated proteins, and conformation-specific antibodies have been developed for protein aggregators. However, the myriad of challenges associated with antibody-based detection and failures of a number of clinical trials have spurred renewed efforts in developing small-molecule probes. Several amyloid plaque-specific positron emission tomography (PET) imaging probes have been reported, including Pittsburgh compound B, [18F] florbetapir, and [18F] flutemetamol. Although these compounds are capable of detecting fibrillar aggregates of the amyloid-β protein (Aβ) that constitute the amyloid plaques in the brain, they are of limited clinical use. These probes are insensitive toward neurofibrillary tangles composed of fibrillar aggregates of the tau protein, undermining their diagnostic value as AD entails a complex regional localization of both Aβ and tau deposits.

The shortcomings of existing PET probes partly stem from the molecular scaffolds from which they are derived, primarily the 2-phenylbenzothiazole dye thioflavin T (or the mixture of related dyes “thioflavin S”), commonly used for the histologic detection of amyloid. All amyloid-binding agents share a common rigid or semiflexible “conjugated rod” motif, which allows them to favorably bind to the hydrophobic surface grooves on amyloid fibrils. When used for fluorescence imaging, these ligands primarily function on a “molecular rotor” basis, in which fluorescence shift or enhancement occurs because of planarization of the conjugated region and prevention of fast nonradiative decay by intramolecular rotation.

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improved sensing capabilities are thus urgently needed to unravel the underlying pathogenesis of amyloid-related diseases. While radiopharmaceutical tracers have the advantage in the preclinical and clinical setting, optical sensors are uniquely suited to broad adoption because of the wide availability of instrumentation for imaging and spectroscopy. Along with high sensitivity for the aggregated protein conformation and a large increase in brightness upon binding, the critically important property of a sensor is its ability to detect the aggregates formed from a wide set of promoter proteins.

Recently, we have reported the use of novel oligo(phenylene ethynylene) electrolytes (OPEs) as optical sensors for amyloid fibrils formed from hen egg-white lysozyme. The fibrils formed by incubating the lysozyme under acidic conditions are structurally and morphologically indistinguishable from those formed from the disease-associated proteins. Lysozyme oligomers and fibrils have also been shown to be cytotoxic toward the human neuroblastoma cells, indicating that the aggregates of lysozyme recapitulate the biological activities of the known disease-associated proteins. Four OPEs (Table 1, compounds B4, B5, B6, and OPE1), differing in charge and size, were tested and found to selectively detect lysozyme fibrils in vitro by binding-activated increase in the fluorescence yield, with low micromolar affinity and high selectivity for the amyloid conformation of lysozyme over the

Table 1. Structures of the 18 PPE-Based Oligomers and Polymers Used in This Study

| BackBone Structural Class | Side and end chains, and number of repeat units (n) | Compound Name |
|--------------------------|---------------------------------------------------|---------------|
| **Oligomeric Compounds** |                                                   |               |
| ![Chemical Structure](image) | ![Chemical Structure](image) | A1            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | A2            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | A3            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B1            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B2            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B3            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B4            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B5            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B6            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B7            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B8            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B9            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B10           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B11           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B12           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B13           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B14           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B15           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B16           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B17           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | B18           |
| ![Chemical Structure](image) | ![Chemical Structure](image) | OPE1          |

Polymeric Compounds

| ![Chemical Structure](image) | ![Chemical Structure](image) | C1            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | C2            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | C3            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | C4            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | C5            |
| ![Chemical Structure](image) | ![Chemical Structure](image) | C6            |
native conformation of protein.23 On the basis of spectral properties and previous work in analogous systems, we posit that the fluorescence turn-on mechanism is a combination of OPE backbone planarization, reduction of solvent-mediated quenching of the OPE ethyl ester end groups, and the formation of chiral J-type OPE aggregates templated on the lysozyme fibril surface.23

To validate that the OPEs are useful optical sensors that can selectively detect the fibrillar amyloid conformation of proteins independent of the properties of the underlying protomer, we report here the binding and fluorescence of the OPE compounds to the fibrils formed from another model amyloid protein, bovine insulin. Similar to lysozyme, insulin readily forms fibrillar aggregates when incubated under acidic and high-temperature conditions. Moreover, while lysozyme is positively charged at neutral pH, insulin is negatively charged at the same pH. The common fibrillar morphology of lysozyme and insulin aggregates, combined with large differences in the charge states of the proteins, allowed for testing the selectivity of the OPE sensors for fibrillar over monomeric protein conformations. In this study, we additionally tested a small library of oligomeric and polymeric phenyleneethynylenes (PPEs) (Table 1) to further probe the effects of charge and substituents on fluorophore amyloid binding and recognition and compare the amyloid interactions of the conjugated small molecules of varying size and conjugated polymers. The results of this study support previous research into the molecular mechanism of amyloid detection by small molecules and provide insights into new properties that may allow enhanced detection of these important analytes.

■ RESULTS AND DISCUSSION

The library of 18 conjugated compounds tested (Table 1) comprises a variety of molecular weights, charge densities, water solubilities, and types and geometries of functional groups, all based on the linear PPE molecular scaffold. Twelve oligomeric PPE compounds with well-defined molecular weights were used. Compounds A1, A2, and A3 are cationic and relatively small molecules with no side chains and charged end groups. The B series compounds and OPE1 have side chains that are either cationic (B1−B8) or anionic (OPE1) and have different end groups and a varying number of repeat units (n = 1, 2, or 3). The six polymeric compounds have high and not well-defined molecular weights (n < 1000) and vary in the conjugated backbone structure and cationic side chains. The oligomeric compounds are water-soluble and well-dispersed in aqueous solutions. Although the charged polymers are also water-soluble, they form aggregates in aqueous solutions.26 From this structural diversity, we expect to validate and glean some guiding principles for functional amyloid sensor molecules and investigate structure−property relationships for binding-activated photophysical changes.

To test the efficacy of the library of PPE-based compounds to selectively detect protein aggregates, the excitation and emission fluorescence spectra of each compound were measured after incubation with the monomeric or fibrillar hen egg-white lysozyme and bovine insulin. As these two proteins differ in their charge, hydrophobicity, and surface residues, they are well-suited to test the robustness of amyloid sensing. The amyloid aggregates of these two proteins were prepared by the incubation of the stirred protein solutions at low pH and high temperatures for 24 h. Lysozyme23 and insulin amyloid aggregates were thioflavin T-positive (Figure S1). Far-ultraviolet (UV) circular dichroism (CD) spectra of the protein solutions (Figure 1b,d) confirmed the transformation of the primary α-helical native structures of both proteins, as shown by the negative bands at 208 and 222 nm, into the characteristic

Figure 1. AFM images of insulin (a) and lysozyme (c) fibrils deposited on mica (imaged in air by the tapping mode). Far-UV CD spectra of insulin (b) and lysozyme (d) monomers and amyloid fibrils indicate the loss of native α-helical structures to the formation of β-sheet secondary structures. The error bars in the CD spectra are standard errors of replicate measurements of the same sample.
β-sheet structures of amyloid fibrils, as shown by the negative band at 218 nm. Atomic force microscopy (AFM) images of the incubated protein solutions deposited on mica (Figure 1a,c) showed short fibrillar aggregates, with individual linear structures composed of two or more twisted subfibrils, and average dimensions of about 100 nm long and 20 nm wide. The short fibril morphology is consistent with incubation conditions where low pH and high temperature caused fast fibril nucleation and growth, and agitation caused competing fibril fragmentation.

The in vitro formed amyloid aggregates of lysozyme and insulin were tested against the 18 different PPE dyes with a significant structural heterogeneity (Table 1). The fluorescence emission and excitation spectra of the dyes alone in buffer, with a monomeric protein, and with an aggregated protein were recorded, with the main readout of interest being the changes in emission intensity and the shifts in excitation or emission wavelengths. Complete spectral data can be found in the Supporting Information. To ease the evaluation of amyloid sensing, that is, whether a compound exhibits selective sensing of the fibrillar conformation of a protein over its native structure, the fluorescence spectral data were processed and reduced to a factor quantifying the selective amyloid-sensing performance, the amyloid detection factor (ADF), using eq 1

$$ADF = \frac{F_{\text{dye+monomer}} - F_{\text{dye+brillar}}}{F_{\text{dye in buffer}}}$$

where $F_{\text{dye+monomer}}$ is the integrated fluorescence signal of the dye in solution with the native protein, $F_{\text{dye+brillar}}$ is the fluorescence signal of the dye in solution with amyloid aggregates of the protein, and $F_{\text{dye in buffer}}$ is the fluorescence signal of the dye alone in buffer. Integrated rather than the peak fluorescence intensity values were used to better model the situation in the microscope, where all photons that pass through the filter set are integrated at the detector. As defined, the ADF value is thus indicative of the ability of a dye to selectively detect the amyloid conformation of a protein from its native conformation relative to the background fluorescence of the dye in buffer. As such, the ADF values near zero indicate that the dye does not distinguish between the monomeric and fibrillar states of a protein. This effect is due to the lack of dye binding or binding without a concomitant increase in the fluorescence signal. The negative ADF values indicate that the dyes exhibited higher fluorescence in the presence of protein monomers than protein fibrils; the dyes are thus selective sensors for the monomeric protein conformation. The positive ADF values indicate that the dyes yielded higher fluorescence in the presence of fibrillar aggregates of a protein than monomers; these dyes are thus selective sensors for the protein amyloid state and potentially useful sensors for protein aggregate biomarkers. Furthermore, the magnitude of the ADF value indicates the extent by which the dye distinguishes the fibrillar versus the monomeric state of the protein over background fluorescence of the dye. The larger the ADF value, the better the sensor signal. This data processing and reduction approach allowed us to easily identify compounds in the library that exhibited useful photophysical changes when interacting with amyloid. Figure 2 summarizes the ADF values obtained for the 18 conjugated compounds for both lysozyme and insulin proteins.

As shown in Figure 2, many of the tested compounds gave rise to the ADF values close to 0 (within the gray “insensitive” zone in the figure), indicating that either these compounds did not bind to the proteins or any interaction with the proteins did not lead to changes in fluorescence. The oligomeric compounds A1, A2, and A3 had significant background fluorescence in buffer and did not exhibit increased fluorescence in the presence of either monomeric or fibrillar lysozyme or insulin (Figure S4). Most of the polymeric compounds, C1, C3, C4, C5, and C6, also gave rise to the ADF values close to 0. These compounds have significant background fluorescence in buffer, and although some compounds exhibited increases in fluorescence when added to protein monomers or fibrils, their fluorescence signals were not useful as they were not selective for either the fibrillar or the monomeric state of lysozyme or insulin (Figures S8 and S9). However, enhanced fluorescence was observed for many compounds when they were mixed with protein monomers or fibrils as the dyes gave rise to large positive ADF values (fibril selective) or large negative values (monomer selective), including most oligomeric compounds in the B series and the polymeric compound C2. A few of the dyes were effective sensors for the fibrillar conformation of one protein (positive ADF values) and for the monomeric conformation of the other protein (negative ADF values), including B3, B5, and B6. Importantly, two small dyes, cationic B4 and anionic OPE1, are identified as selective sensors for both lysozyme and insulin fibrils, as the positive ADF values were obtained for each dye for both proteins. As the magnitude of the ADF values for OPE1 was larger than that for B4, the anionic OPE1 compound appears to be the more sensitive selective amyloid sensor from the library of PPE compounds screened. In addition to identifying useful sensors, other useful observations about the effects of substituents on the conjugated backbone, charge, and molecular size on the amyloid-sensing effectiveness of the dyes can be gleaned from the ADF results.

The effect of substituents on dye sensing is especially stark. Except for the polymers (C2), the only dyes with significant positive ADF values, that is, selective detectors of amyloid, were B4, B5, B6, and OPE1, all bearing ethyl ester end groups. This small structural substitution has profound effects on the photophysical properties of the compounds. Whereas the fluorescence quantum yields of H- and ester-terminated dyes such as B1, B2, B3, and B8 were similar in methanol and water, the quantum yields of the ethyl ester terminated dyes B4, B5,
Table 2. Quantum Yield of Fluorescence (φfl) Values of Select PPE Compounds in Methanol (MeOH) and Water (H2O)

| compound | φfl (MeOH) | φfl (H2O) |
|----------|------------|------------|
| B1       | 0.66 ± 0.02 $^a$ | 0.64 ± 0.02 $^a$ |
| B2       | 0.73 ± 0.02 $^a$ | 0.13 ± 0.02 $^a$ |
| B3       | 0.72 ± 0.02 $^a$ | 0.040 ± 0.02 $^a$ |
| B4       | 0.75 ± 0.02 $^a$ | 0.233 ± 0.001 $^a$ |
| B5       | 0.71 ± 0.01 $^a$ | 0.039 ± 0.001 $^a$ |
| B6       | 0.7 ± 0.02 $^a$ | 0.069 ± 0.001 $^a$ |
| B7       | 0.66 ± 0.02 $^a$ | 0.64 ± 0.02 $^a$ |
| B8       | 0.7 ± 0.01 $^a$ | 0.74 ± 0.04 $^a$ |
| OPE1     | 0.75 ± 0.02 $^a$ | 0.023 ± 0.02 $^a$ |

$^a$From ref 36. $^b$From ref 40.

B6, and OPE1 are dramatically reduced in water compared to those in methanol (Table 2), providing a mechanism for the very effective at selectively detecting lysozyme fibrils, for which large increases in fluorescence intensities were observed when the compounds were mixed with lysozyme fibrils and only small increases were observed when the compounds were mixed with lysozyme monomers. However, sensing selectivity was reversed when the dyes were mixed with insulin fibrils: large increases in fluorescence intensities were observed when the compounds were mixed with insulin monomers and only small or no increase was observed when the compounds were mixed with insulin fibrils. The significant increases in the fluorescence of the B5 and B6 compounds to insulin monomers can be attributed to binding because of attractive Coulombic interactions between the cationic compounds and a negatively charged insulin monomer at neutral pH. This long-range attractive interaction allows the dye and an insulin monomer to approach close enough for the dye to bind to the hydrophobic pockets on the protein surface. Once bound, the ethyl ester terminated compounds exhibit hydrophobicity-induced unquenching, yielding large increases in the fluorescence intensity. Overall, the larger cationic ethyl ester terminated B5 and B6 compounds are good sensors as the binding of these compounds to lysozyme fibrils and insulin monomers resulted in large increases in the fluorescence signal. However, they are not suitable amyloid sensors, as their sensing is protein-specific and they did not detect the amyloid conformation of the insulin protein (Figure 3).

On the basis of Coulombic interactions, the anionic dyes B7, B8, and OPE1 are expected to exhibit favorable binding to the positively charged lysozyme monomers at neutral pH. However, significant enhancement in fluorescence was not observed (Figure S7). Compounds B7 and B8 do not possess the ethyl ester end groups that give rise to fluorescence quenching in aqueous solutions, as shown by the comparable fluorescence quantum yield values of these two compounds in methanol and water (Table 2). Thus, even if these compounds bind to lysozyme based on favorable Coulombic interactions, the dyes are not useful sensors as the binding is not accompanied by increases in the fluorescence signal. By contrast, the fluorescence of B7 and B8 decreased in the presence of lysozyme monomers and fibrils compared to the fluorescence of the dye in buffer (Figures 4c and S7). The best selective amyloid sensor identified in this screening study is the small anionic OPE1 compound as it exhibited increased fluorescence in the presence of both lysozyme and insulin fibrils (Figures 3 and 4). Importantly, only small increases in the fluorescence of OPE1 were observed in the presence of a monomeric lysozyme or insulin. On the basis of Coulombic interactions, the anionic OPE1 compound is expected to favorably bind to the cationic insulin monomers. However, very little binding between OPE1 and insulin monomer was observed. Although this effect cannot yet be fully explained, we postulate that this effect is due to the higher solvation energy of the anionic −SO$_3^-$ group as compared to that of the −NMe$_3^+$ group in B5 and B6, reducing the propensity of the OPE1 molecule to desolivate and bind to hydrophobic patches on the protein monomer. Experimental$^{29}$ and theoretical$^{30}$ studies show that the Gibbs free energies of the aqueous solvation for −SO$_3^-$ versus −NMe$_3^+$ differ by several hundred kcal/mol because of the larger size of the anion and its greater number of H-bond acceptors.

Size is the third major characteristic affecting the dye performance. The small-end-substituted dyes A1, A2, and A3, although perhaps capable of binding appropriately, lack a
mechanism for enhancement in the fluorescence signal upon binding. In fact, these molecules have been shown to lose the fluorescence yield when complexed because of the formation of H-type aggregates.\textsuperscript{31} Polymers (compounds C1–C6), for the most part, did not perform well either. Increases in fluorescence were observed when a few of the cationic polymers were added to anionic lysozyme fibrils (Figure S8). However, as no fluorescence increases were observed when the compounds were added to insulin fibrils, these dyes were not useful amyloid sensors. Performance of these polymeric compounds was likely affected by the size mismatch with a binding site or solubility problems. Because of their extended hydrophobic backbone, many of these polymers are highly self-aggregated in aqueous solutions, resulting in low solubility and depressed emission intensity.\textsuperscript{26} These high-molecular-weight polymers are unlikely candidates for tissue stains or in vivo fluorescent probes because they are unlikely to transit membranes readily or diffuse very far. The series of cationic ethyl ester terminated oligomeric B4, B5, and B6 compounds provide an extremely useful insight into size effects on the amyloid detection performance. These three dyes vary only in the number of repeat units, and their ADF values of lysozyme sensing shown in Figure 2 (gray bars) show a clear optimum at the size of B5 ($n = 2$), with five phenyl rings in the PPE chromophore. B4 ($n = 1$) is too small to have enough exposed hydrophobic surface to bind strongly to yield large increases in fluorescence, and B6 does not have enough of the chromophore bound to protein to elicit unquenching of the ethyl ester end groups, or possibly the end groups are not sufficiently shielded from water in the bound state. The typical twisted amyloid structural model, which places the hydrophobic binding grooves at an angle to the fibril axis, indicates the possibility of a maximum effective size for a linear chromophore to bind: if the chromophore is too long, it cannot conform to the curvature of the binding site. Possibly longer chromophores may be effective if they have a flexible backbone structure.\textsuperscript{32}

Overall, our results show that small changes in the dye structure can have a profound effect on the amyloid sensor performance. The least effective sensors were the dyes with charged groups on the ends (A1, A2, and A3) and those with charged groups on the sides of the chain and no moiety on the terminal phenyl rings (B1, B2, and B3). The cationic ester-terminated dyes (B4, B5, and B6) were more responsive to the presence of protein but had significant drawbacks. These compounds interacted with and exhibited enhanced fluorescence with both native and fibrillar insulin, as indicated by their negative ADF values in Figure 2 (see also Figure S6), and the longer dyes (B5 and B6, with $n = 2$ and 3 repeat units, respectively) had some selectivity for lysozyme amyloid, but overall none of these dyes performed well relative to others in the screening set.

The sole dye found to perform effectively as a selective sensor for the amyloid conformation of the two model proteins was the small anionic OPE1 that vastly outperformed even very close structural analogues. Fluorescence of the anionic OPE1 was low in the presence of both cationic lysozyme monomers and anionic insulin monomers (Figure 4a,b). In the presence of fibrillar aggregates, OPE1 exhibited large increases in the binding-activated fluorescence. Thus, OPE1 is a protein-conformation selective sensor and not a protein-specific sensor. OPE1 binds to lysozyme and insulin amyloid fibrils with dissociation constants ($K_d$) in the low micromolar range (Table 4).
dissociation constants for thioflavin T binding to insulin and lysozyme amyloid vary but range between 500 nM and 23 μM for insulin fibrils and between 150 nM and 25 μM for lysozyme fibrils, putting OPE1 well in the useful range of affinities. Induced CD was also observed for OPE1 with insulin and lysozyme amyloids (Figure S2), indicating that the dye binds as a chiral complex, possibly a chirally biased J-type dimer. The peak of the emission spectrum for OPE1 bound to the insulin amyloid is blue-shifted with respect to the emission peak of OPE1 bound to the lysozyme amyloid (Figure 4b), indicating that the J-aggregate character is more dominant in the insulin-bound population. Comparison of the fluorescence spectra of OPE1 with those of its structural analogue B7 (Figure 4, top and bottom) demonstrates the importance of a substituent on effective sensing. B7 and OPE1 are identical except that OPE1 bears ethyl esters on both ends of the conjugated backbone, whereas B7 does not. B7 loses some fluorescence yield in the presence of lysozyme monomers or fibrils and is completely insensitive to insulin in any form. The anionic OPE1 is the only molecule with positive ADF values for both lysozyme and insulin amyloid.

## CONCLUSIONS

This study evaluated a small library of 18 different fluorescent compounds based on a p-PPE backbone for their ability to selectively detect the amyloid aggregate conformers of two model amyloid proteins. Most of the compounds showed some nonspecific binding to protein monomers, and some were able to detect amyloids of either insulin or lysozyme, but only one compound performed effectively with both proteins: a shorter compound with anionic sulfonate solubilizing groups and ethyl ester end groups (Figure 5).

The very small and hydrophobic end-functionalized compounds (A1, A2, and A3) did not effectively sense the amyloid state, possibly because they can nonspecifically interact with many binding sites. These compounds have been shown to have high toxicity to bacterial cells and to inactivate viruses, suggesting that they can interact destructively with proteins. We might have expected that the very large repeating polymeric dyes would sense amyloids with high selectivity because of their long linear chains binding to equally long linear aggregate structures. The results show that this was not the case—some polymers bound to amyloids with moderate sensitivity, but none bound well to both insulin and lysozyme amyloid.

Exchanging cationic quaternary ammonium groups for anionic sulfonate groups has multiple profound effects on the mechanics of binding, as evidenced by the formation of less emissive states by B7 and B8 dyes and the very different behavior of OPE1 and B5 (Figure 5). For small molecules, the anionic groups seem to reduce nonspecific binding to monomers appreciably, especially for insulin. We postulate that this effect is due to the higher solvation energy of the —SO3 group as compared to that of the —NM4 group, reducing the propensity of the molecule to desolvate and bind to hydrophobic patches on the protein monomer.

The results of this study have provided some structural insights into the binding and fluorescence sensing of amyloids by PPE-based dyes. For this class of compounds, restriction of intramolecular motion by binding alone does not seem to be sufficient for the sensitive monomer-independent detection of amyloids—that is, they do not function like a molecular rotor. A secondary mechanism of binding-activated quantum yield increase (in this case, unquenching by reduced water accessibility) is necessary to produce an effective sensor. Overall, anionic structures are less prone to nonspecific binding and more broadly biocompatible, and smaller size seems to be crucial for effective interaction. These results will provide guidelines for future investigation of amyloid-binding fluorogenic dyes.

## EXPERIMENTAL SECTION

Synthesis of PPE dyes was reported previously. Hen egg-white lysozyme and bovine insulin were obtained as lyophilized powders from Sigma-Aldrich (St. Louis, MO) and used without further purification. Purified and reagents used to make buffers were obtained from Sigma-Aldrich. Thioflavin T (ThT) was obtained from VWR International (Radnor, PA). Water used was purified to a resistivity of 18.2 MΩ at 25 °C (Millipore Synergy UV purification system, EMD Millipore, Billerica, MA). All experiments were performed in duplicate, and the mean values were reported with standard error. Paired t-test was carried out to assess the statistical significance of the difference between the PPE fibril fluorescence and the PPE monomer fluorescence. The spectra reported are a single representative experiment.

Lysozyme and insulin fibrils were prepared by dissolving each protein at 5.46 mg/mL in 10 mM sodium citrate buffer at pH 3 with 0.1 M NaCl and by incubating at 70 °C for 12 h with 250 rpm magnetic stirring. Extinction coefficients at 280 nm of 1.0 L/(g·cm) for insulin41 and 2.63 L/(g·cm) for lysozyme42 were used to determine the concentrations. Both protein solutions, initially clear, formed a cloudy precipitate within an hour of

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**Table 3. Binding Constants (K_d) of OPE1 to Lysozyme and Insulin Amyloid. K_d Values of Thioflavin T Are Included as a Reference**

| Protein        | K_d of OPE1 (μM) | K_d of thioflavin T (μM) |
|-----------------|------------------|--------------------------|
| insulin amyloid | 2.0 ± 0.73       | 0.5–23                 |
| lysozyme amyloid| 0.75 ± 0.19      | 0.15–25                |

*From ref 33. From ref 34.
heating. For ThT assay, protein and ThT stock solutions were mixed in pH 7.4 10 mM phosphate buffer (PB) to a final concentration of 5 μg/mL protein and 20 μM ThT, and the emission spectra were obtained at 440 nm excitation (Figure S1). Presence of amyloids was confirmed by the direct observation of fibril morphology with AFM on mica in air (Figure 1).

In the wells of a 96-well plate, a monomeric protein, an amyloid, or a buffer blank was diluted with dye stocks into PB to a final concentration of 2 μM OPE or 1.88 μg/mL PPE (the same monomer concentration as the intermediate-size OPEs) and 5 μg/mL protein. The final sample volume was 200 μL, and each well was prepared in duplicate. The absorption and emission spectra of the samples were then measured on a plate-reading spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA). For the higher resolution evaluation of spectral changes, a steady-state cuvette-reading fluorimeter (PTI QuantaMaster 40, HORIBA Scientific, Edison, NJ) was used. The same concentration and sample volumes were prepared and transferred to a small-volume quartz cuvette (Starina Cells, Atascadero, CA), and the excitation and emission spectra were obtained. To determine the secondary structures of a protein (Figure S3), CD spectra of the protein solutions through a 30 kDa molecular weight filter (EMD Millipore, Billerica, MA). The same concentration and sample volumes were used. The same concentration and sample volumes were prepared and transferred to a small-volume quartz cuvette for measuring the absorbance at 280 nm. The fluorescence of the samples containing a final fibril concentration (500 nM) and a range of OPE concentrations was measured using a spectrofluorometer. Peak fluorescence values versus the OPE concentration was subsequently plotted and fitted to the Hill equation using OriginPro 9 to obtain the equilibrium dissociation constant (K_d) values.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00231.

Thioflavin T assays, induced dye CD measurements, AFM images, plots of binding assays, and complete spectral data (PDF)

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**Notes**

The authors declare no competing financial interest.

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