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Aza-BODIPY: improved synthesis and interaction with soluble A β 1-42 oligomers

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Abstract

Dye-binding assays that are used to evaluate anti-aggregation ability of small molecule inhibitors towards amyloids are known to be prone to false-positive effects due to spectral overlaps between the dye and the inhibitor. Aza-BODIPY dye, which has both excitation and emission maxima above 600 nm, exhibits a significant increase in its fluorescence intensity in the presence of soluble oligomers of A β 1-42. These results indicate that aza-BODIPY could serve as a near-IR probe for detecting conformational changes of A β 1-42 soluble oligomers *in vitro*, and it should eliminate false-positive effects that are associated with currently utilized thioflavin T-based dyes. In addition, a facile synthesis of aza-BODIPY has been developed, which might further expand the applications of this dye.

Keywords

aggregation; amyloid; circular dichroism; fluorescent dye; microwave synthesis

Alzheimer's Disease (AD) is an age related cognitive disorder concomitant with the decline of learning and memory, resulting from a disruption of neuronal communication and cell death. It is characterized by the formation of neurofibrillary tangles and amyloid β -protein (A β) based deposits or plaques.¹ It was long suggested that these insoluble fibrils were the pathogenic species in AD.² However, recent studies have shown that soluble oligomeric A β aggregates are actually responsible for the loss of neuronal function.^{3–7} Furthermore, the conformational transition from an unordered structure to an ordered, β -sheet like A β species is believed to be responsible for the neurotoxicity.^{8,9} Detection of these oligomers, as well as their conformational changes and associated aggregation is an important, yet not well developed, area of research.¹⁰

Dye-binding assays, such as those utilizing thioflavin T (ThT), are among the most widely used tools to probe the *in vitro* aggregation of amyloidogenic biomolecules and for the evaluation of small molecule inhibitors of amyloid aggregation and fibrillization.^{11,12} Due to practical considerations, specifically the ease of manipulation and high throughput screening possibilities, dye-binding assays often serve as the initial test of anti-aggregation ability of small molecules. However, a number of reports have suggested that these dye-binding assays could be prone to false positive effects when assessing inhibitors' potential towards A β peptides.¹³ Spectral overlap of the inhibitor molecules with the dye, competitive binding

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Supplementary data

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and a high concentration of the dye which could compromise the amyloid self-assembly, are among major concerns associated with these assays.

In regard to ThT, it has been shown that small molecules with intrinsic fluorescence and/or overlapping absorption spectra can interfere with ThT fluorescence and may lead to false positives in dye-binding assays.^{14–18} In aqueous media, ThT has an excitation and emission maxima at *ca.* 350 and 440 nm, respectively. Upon interaction with β -sheet rich species, the maxima are shifted to 440 and 490 nm. It should be noted that many small molecules have an absorption in the 300–500 nm range, and thus would overlap with ThT, potentially causing false positive effects. Furthermore, many accounts suggest that in the dye-binding assays, an excess of the dye relative to the peptide is required.^{11, 19–21} Arguably, this could perpetuate the competitive binding of the dye with the potential inhibitor leading to false positive effects, or even perturb the self-assembly process. Moreover, large amounts of ThT have been reported to lead to self-quenching.²² Additionally, ThT is reported to form micelles above 4 μ M, which might alter the effective concentration of an inhibitor molecule.²³ Arguably, fluorescent dyes that could interact with A β at substoichiometric amounts, and have excitation and emission above 500–600 nm would avoid the aforementioned issues.

Recently BODIPY dyes have been introduced as viable probes for both soluble and fibrillar amyloid species.^{24–26} However, many BODIPY dyes have absorption and emission characteristics that are only slightly different from ThT, *i.e.*, λ_{ex} *ca.* 500 nm and λ_{em} at *ca.* 530 nm. Consequently, many potential overlaps with small molecule inhibitors are possible. It should be noted that several near-IR BODIPY dyes have been reported, though their ability to act as alternatives to ThT has yet to be explored.²⁷

Alternatively, aza-BODIPY **1** (Scheme 1) also is a near-IR analogue of BODIPY, and has both an excitation and emission well above 600 nm.²⁸ The scope of applications for aza-BODIPY remain largely unexplored, arguably due to the lengthy, multistep synthetic routes.

A typical synthesis of **1** that (Scheme 1)²⁹ utilizes readily accessible reactants and fewer number of steps compared to other reported routes³⁰ still requires several days. Hence, we sought to develop a more facile route to **1**.

Chalcone **2**, which is commercially available, could also be obtained within a few minutes by simply grinding benzaldehyde and acetophenone with NaOH powder using pestle and a mortar.^{31, 32} For the subsequent Henry reaction, which is typically done in refluxing MeOH,²⁹ we found that **3** could be efficiently obtained under solvent-free conditions in 6 hours.

Next, the cyclization step leading to **4** was investigated (Scheme 1). According to literature,²⁹ this step is conducted either neat or in refluxing butanol or ethanol over several days. In an effort to expedite this reaction, we screened several solvents, including dioxane, DMF, DMSO, decanol, hexafluoroisopropanol and trifluoroethanol (TFE). We found that although in all solvents a blue product was obtained (an indication of the formation of cyclized product **4**) after about 8–10 hours, only in TFE a full consumption of the starting material **2** was observed (as assessed by TLC). Thus, it appeared that TFE could significantly accelerate the rate of the aza-tetraphenyldipyromethene **4** formation. Next, in order to reduce the reaction time even further, we examined the synthesis of **4** under microwave (MW) irradiation. The starting material was completely consumed when **3** (67 mM) was MW irradiated for only 30 minutes in the presence of NH₄OAc (137 eq.) in TFE.

However, it should be noted that some inconsistency in the conversion was observed from run to run, most likely due to inefficient stirring of the reaction mixture during the MW irradiation. Thus, we varied the amount of NH_4OAc from 1.0 eq to 137 eq, and we found that the full consumption of **3** could be reproducibly obtained with 62 eq. NH_4OAc in 60 minutes of MW irradiation.

Next, we explored the nature of the ammonia source, *e.g.*, $(\text{NH}_4)_2\text{CO}_3$, $\text{NH}_4\text{CF}_3\text{CO}_2$, triammonium salt of citric acid and diammonium salt of ethylene diamine tetracetic acid. However, it appeared that NH_4OAc was the most efficient source. The isolated yield (after flash chromatography) of **4** was 47%, which is on par with reported yields.²⁹ Formation of a trans conformation of **4**, which is not capable of forming a BF_2 complex, along with multiple intermediates (based on a detailed mechanistic study)³³ accounts for the moderate yields in the formation of **4**.

Complexation of **4** with $\text{BF}_3\text{-Et}_2\text{O}$ is typically done in a solvent over several hours at room temperature.^{29, 34} We found that the BF_2 -moiety could be installed under neat conditions within 90 minutes under reflux. Overall, the developed approach provides a more facile access to **1** as compared to the literature reports.

Next, we investigated the interaction of **1** with A β 1-42 soluble oligomers. Distinct conformations of A β 1-42 were prepared according to published procedures,^{24, 35} and the time dependent conformational transition from unordered to ordered β -sheet rich species was confirmed by circular dichroism (CD) spectroscopy (Figure 1).³⁶

Interactions of dye **1** with distinct conformations of A β 1-42 were investigated by titrating **1** into solutions that contained ordered or unordered A β -soluble oligomers (Figure 2). We found that in the presence of the unordered soluble A β -oligomers, the fluorescence intensity of dye **1** increased *ca.* 6-fold (as compared to the emission intensity of the dye in buffer, in the absence of the A β 1-42 oligomers), while *ca.* 16-fold emission enhancement (F/F_0) was observed when aza-BODIPY **1** dye was added to the solution containing the ordered, β -sheet rich conformation of A β 1-42 (Figure 2, inset).

In addition, the interaction of **1** with ordered A β oligomers appeared to be largely time-independent. The time-dependent behavior of the dye with the unordered oligomers could not be unambiguously assessed, since the peptide is undergoing a time-dependent conformational change.

To further explore the ability of **1** to recognize amyloidogenic species, we prepared soluble aggregates of bovine serum albumin (BSA), as an easily accessible model system.³⁷ Heating BSA for 10 min at 90°C produced a β -sheet rich BSA (Figure 3A). Although the CD spectra of the aggregated BSA did not exhibit the characteristic β -sheet signature with a 215 nm minimum, the deconvolution analysis³⁸ revealed that this heat treatment increased the β -sheet component (calculated as a sum of β -strands and β -turns) from 23 to 45%, while the α -helical content decreased from 60 to 34%. The aggregated BSA was also found to be stable when stored at room temperature over a period of three weeks, as no changes in its CD spectra were noted.

It appeared that interaction of dye **1** with both native and β -sheet rich BSA was time-dependent: the fluorescence of **1** increased over several hours and reached a plateau at 12 hours in the presence of the protein. Therefore, the dye was incubated with both forms of BSA for 12 hours before the emission data were acquired (Figure 3B). About 57-fold fluorescence increase was observed upon binding of **1** to the native BSA, while *ca.* 340-fold

enhancement was detected in the presence of the soluble aggregates of BSA. A more detailed study on the interaction of dye **1** with BSA aggregates will be reported elsewhere.

Overall, the aforementioned results indicated that aza-BODIPY **1** could serve as a probe for detecting conformational changes of A β 1-42 soluble oligomers and also serve as a potential probe for other amyloidogenic forms of biomacromolecules. Importantly, the emission maximum of dye **1** was significantly shifted towards longer wavelengths, *i.e.*, *ca.* 670 nm when bound to a protein (as compared to 490 nm for bound ThT, and 530 nm for some BODIPY dyes). Also, only small concentrations of **1** were required to obtain an appreciable fluorescent enhancement, and thus perturbation of the amyloid self-assembly should be minimal upon addition of **1**. Therefore, this dye could circumvent limitations of currently employed dye-binding assays, and as such, might be suitable for the unambiguous *in vitro* evaluation of small molecule inhibitors of amyloid aggregation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CD spectra of 25 μM Aβ1-42 unordered (blue) and ordered (red) oligomers. Conditions: 10 mM TRIS-NH₄Cl, pH 8.6 buffer, 25 μM Aβ1-42.

Figure 2.

Fluorescence spectra of **1** in the absence (purple) as well as in the presence of the unordered (blue) and ordered (red) Aβ1-42 oligomers. Inset: fluorescence enhancement (F/F_0) of **1** in the presence of unordered (blue) and ordered (red) Aβ1-42 oligomers. Conditions: 10 mM TRIS-NH₄Cl, pH 8.6 buffer, 25 μM Aβ1-42, 0.5 μM **1**, <1% DMSO (v/v). Data are reported as the average of three independent measurements with $\pm$ SD as error bars.

Figure 3.

A: CD spectra of native (blue) and aggregated (red) BSA; conditions: 20 mM TRIS-HCl, pH 7.4 buffer; 91 μ M BSA. **B:** fluorescence spectra of **1** in the absence of BSA (purple), in the presence of native (blue) and aggregated (red) BSA; conditions: 20 mM TRIS-HCl, pH 7.4 buffer; 2 μ M **1**; 91 μ M BSA, 2% DMSO (v/v).

	(a)	(b)	(c)
Ref. 29	CH ₃ NO ₂ , DEA, MeOH, Δ, 24h , 85%	NH ₄ OAc, EtOH, Δ, 48h , 42%	BF ₃ -OEt ₂ O, DIEA, DCM, rt, 24h , 90% [¶]
This work	CH ₃ NO ₂ , DEA, Δ, 6h , 90%	NH ₄ OAc, TFE, Δ, 1h (MW), 47%	BF ₃ -OEt ₂ O, TEA, Δ, 90min , 90%

Scheme 1.

Syntheses of aza-BODIPY **1**; ¶ – the yield for **4** was not reported but rather a range of yields (60–90%) was given for a set of aza-BODIPY dyes. Abbreviations: diethylamine (DEA), diisopropylethylamine (DIEA), trifluoroethanol (TFE), triethylamine (TEA).