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ORIGINAL PAPER

A Spectroscopic Study of 2-[4′-(Dimethylamino)phenyl]-benzothiazole Binding to Insulin Amyloid Fibrils

Catherine C. Kitts · David Anton Vanden Bout

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Abstract The spectroscopic properties of 2-[4′-(dimethylamino)phenyl]-benzothiazole (BTA-2) in solution and in the presence of amyloid fibrils were investigated using absorption and fluorescence spectroscopy. Solution studies show that BTA-2 forms micelles in aqueous solutions, but that the dye can be solvated upon the addition of acetonitrile (CH_3CN). BTA-2 binds to amyloid fibrils in solution leading to a characteristic blue-shift in the emission spectrum and an increase in fluorescence intensity. However, in solutions with increasing CH_3CN concentration, there was a marked decrease in binding of the BTA-2 to fibrils. Studies demonstrating the effect of BTA-2 concentration on binding were performed. A comparison with the standard amyloid fluorescent marker, thioflavin T (ThT), showed that BTA-2 is more fluorescent, making it an excellent dye to label amyloid samples.

Keywords Amyloid fibrils · 2-[4′-(Dimethylamino)phenyl]-benzothiazole · BTA-2 · Fluorescence · Thioflavin T

C. C. Kitts · D. A. Vanden Bout (✉)
Department of Chemistry and Biochemistry, Center for Nano and Molecular Science and Technology, and Texas Materials Institute, University of Texas at Austin,
Austin, TX 78712, USA
e-mail: davandenbout@mail.utexas.edu

Present Address:
C. C. Kitts
Department of Physical Chemistry,
Chalmers University of Technology,
Kemivägen, 10,
Göteborg 41296, Sweden

Introduction

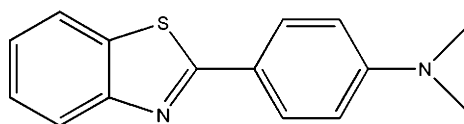
Amyloidosis is associated with over 20 different neurodegenerative diseases, such as Alzheimer's (AD), new variant Creutzfeldt-Jakob, Huntington's, and Parkinson's diseases [1–9]. Specifically, AD is the sixth leading cause of death in the United States for people over 65 years of age [10] and currently has no cure. Therefore the early detection of AD and other neurodegenerative diseases is necessary in order to better understand the progression of these diseases and also to determine a possible treatment [11]. Scientists have been trying to develop sensors [12] or synthesize biomarkers [13] in order to make early detection more attainable. Researchers have found that aggregated proteins, called amyloid fibrils, are present in these diseases, yet the mechanism of formation is still highly debated and a hot topic of research [14, 15]. Amyloid fibrils occur when normally soluble proteins self-assemble into insoluble, highly ordered aggregates [8, 16].

Since the cause of amyloid fibril formation is unknown, scientists have extensively studied their structure to better understand how and why they form. The structure of the amyloid fibril has been found to consist of anti-parallel pleated β-sheets that are oriented perpendicular to the fibril axis [3, 6, 8, 15, 17–20]. These amyloid fibrils are not limited to proteins that cause neurodegenerative diseases, but are also form from proteins found in other organs. This suggests that amyloid fibrils formation is related to the polypeptide backbone of proteins and that proteins can all potentially form amyloid fibrils under certain conditions [6, 8, 21] or by design [22]. Although amyloid fibrils are composed of repeat units of the same protein, amyloid fibrils comprised of different proteins exhibit similar properties (i.e. fibril length and width) [23].

Spectroscopic methods, such as staining the amyloid fibrils with a fluorescent dye [23–38], can be used to gain insight into their structure and formation. The most widely used dyes are Congo red (CR) and thioflavin T (ThT), which when bound to amyloid fibrils and have distinct properties [23–35]. Although these dyes have been extensively studied, they both contain charged groups that limit brain permeability which is important for neurological studies [13, 14]. Currently, scientists are trying to develop fluorescent biomarkers that will bind to amyloid fibrils and can cross the blood-brain barrier in order to utilize non-invasive in vivo imaging techniques [39]. There is a potential for neutrally charged derivatives of CR and ThT to have an increased binding to amyloid fibrils which would increase the permeability into the brain for in vivo imaging [40].

One family of dyes that shows promise for fluorescence imaging techniques is the benzothiazole dyes, which are structurally similar to the extensively researched ThT, which exhibits an enhanced fluorescence signal when bound to amyloid fibrils [23, 31]. Klunk et al. reported on several neutrally charged ThT derivatives and found that they had a higher binding affinity to amyloid fibrils compared to ThT [13]. However, these dyes are relatively new and less extensively characterized. Since research has focused on designing new biomarkers for in vivo imaging, these dyes also show promise for studying amyloid fibrils using absorption and fluorescence spectroscopy. The benzothiazole dye specifically used in this research was 2-[4′-(dimethylamino)phenyl]-benzothiazole (BTA-2) (Scheme 1).

In this paper, we will discuss the spectroscopic properties of BTA-2 in solution, both free and in the presence of insulin amyloid fibrils. Bovine insulin was used as a model because it is a well characterized protein that readily forms amyloid fibrils [35, 41]. The aim of this work is to characterize BTA-2 in solution and bound to amyloid fibrils using absorption, fluorescence, and anisotropy fluorescence spectroscopy. BTA-2 was found to be insoluble in H_2O, but was soluble in acetonitrile (CH_3CN); CH_3CN was chosen as the solvent for the current work based on previous studies of amyloid fibrils in aqueous solutions that were made into films [35]. It should be noted that if using BTA-2 for in vivo imaging that DMSO is a more suitable solvent because of its lower toxicity to cells than CH_3CN.[13] Two distinct solutions were compared. The first solution contained only 2% acetonitrile (CH_3CN)



Scheme 1 2-[4′-(dimethylamino)phenyl]-benzothiazole (BTA-2)

in pH 2 water, while the second solution was a 1:1 mixture of water and CH_3CN. Using the same solvent mixture, BTA-2 was characterized in the presence of amyloid fibrils; however, in the 1:1 mixture of water and CH_3CN, it was determined that BTA-2 did not bind to the fibrils. Therefore a series of experiments were performed varying the CH_3CN concentration in order to determine the effect of incorporating an organic solvent has on binding. Also, how changing the BTA-2 concentration affects binding. Finally, BTA-2 was compared to ThT using fluorescence spectroscopy, free and in the presence of fibrils, to quantify if BTA-2 was a more fluorescent marker than ThT.

Experimental

Materials

Insulin from a bovine pancreas was used as purchased from Sigma-Aldrich and was stored at −20°C in a desiccator. ThT dye was used as purchased from Sigma-Aldrich. BTA-2 was synthesized as described in literature by Alagille et al. [42]. The resulting dye was further purified as describe in Kitts et al.[35]

Fibrils were prepared by dissolving insulin in pH 2 water (5 mg/mL). The solution was filtered through a 0.2 μm filter and then the solution was heated at 60°C for 24 h. After heating, the fibrils were centrifuged using an Eppendorf 5415R centrifuge at 3000 rpm for 2.5 min to remove any globular artifacts. The supernatant containing the fibrils was removed and saved for later use. Solutions were stored in a refrigerator (~10°C) until needed.

BTA-2/fibrils solutions

Fibril solutions for BTA-2 absorption and fluorescence studies were prepared in two ways. The first solution was prepared by diluting an aliquot (100 μL) of the stock fibril solution with 880 μL of pH 2 water and then adding an aliquot (20 μL) of 0.8 mg/mL (3.15 mM) BTA-2 in CH_3CN. The second fibril solution was made using the same amount of stock fibril solution and BTA-2, but was diluted with a 1:1 mixture of nanopure water: CH_3CN. Two similar solutions were made that contained only the BTA-2 without the fibrils. Solutions used for how CH_3CN affects binding of amyloid fibrils varied the amount of CH_3CN, but maintained the same concentration of BTA-2, as stated above, in each solution. The solutions used for how the concentration of BTA-2 affects binding only varied the amount of BTA-2 present, but had a constant amount of CH_3CN, fibrils, and water present in all the solutions. Solutions made with ThT were made in the following way:

an aliquot of 20 μL of 0.8 mg/mL (2.51 mM) ThT in CH_3CN and an aliquot of 100 μL of stock fibril solutions was diluted with 880 μL of pH 2 water.

Spectroscopy

Absorption spectra were obtained using a Beckman DU 7400 UV-Visible Diode-array spectrophotometer. Spectra were collected using a 1.4 mL volume Starna quartz cuvette with a path length of 1 cm (this cuvette was also used for fluorescence spectroscopy). Fluorescence and anisotropy measurements were acquired using a Photon Technologies International Quanta Master Model C Cuvette based scanning fluorimeter. Bandpass filters of appropriate wavelength were used to ensure a monochromatic excitation beam. Long pass filters were used in the emission path to help eliminate scattered excitation light. For anisotropy measurements, polarizers mounted on a rotation stage were placed in the excitation and emission paths. The following polarizations were collected: HH, HV, VH, VV (H=horizontal polarization, V=vertical polarization), where the first letter denotes the polarization of the excitation and the second letter is the polarization of the emission. The G-factor (G) of the instrument was determined by dividing the intensity of the HV polarizations by the intensity of the HH polarizations for each wavelength. The fluorescence anisotropy, r, was calculated for each wavelength using the following equation:

$$r = \frac{I_{VV} - (G^* I_{VH})}{I_{VV} + (2^* G^* I_{VH})}$$

Results and discussion

BTA-2 was studied using absorption and fluorescence spectroscopy to probe its spectral properties in solution and in the presence of amyloid fibrils. Absorption, excitation, and emission spectra were taken of free BTA-2 in pH 2 water (2% CH_3CN) (BTA-2 concentration is 62.90 μM) (Fig. 1). The absorption spectrum of the BTA-2 in pH 2 water had a single absorption peak with a maximum at ~430 nm. The fluorescence emission spectrum, acquired with an excitation of 430 nm, showed a single peak at ~475 nm. The fluorescence excitation spectrum was collected for emission at 500 nm. In contrast to the absorption spectrum, it showed two distinct excitation peaks, one at 370 nm and the other at 430 nm. The smaller peak at 430 nm corresponded to the observed maximum in the BTA-2 absorption. However, the larger peak at 370 nm did not correspond to any feature in the absorption spectrum. Since the excitation spectrum's larger

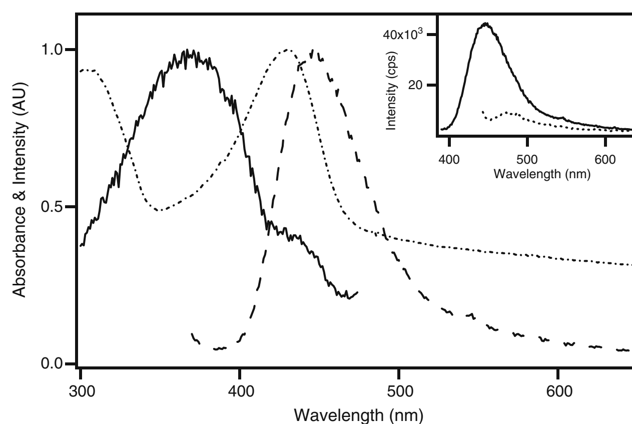


Fig. 1 Normalized absorption (*dotted-dashed line*), excitation (*solid line*), and emission (*dashed line*) spectra of BTA-2 in pH 2 water. Inset is the emission spectra of BTA-2 in pH 2 water excited at 430 nm (*dotted line*) and excited at 360 nm (*solid line*)

peak was at 370 nm, an emission spectrum was acquired with 360 nm excitation. This emission spectrum had a single peak at 450 nm, and was notably blue-shifted from the 475 nm peak observed when the sample was excited at 430 nm. The emission at 450 nm also had a 5 fold increase in intensity compared to the emission when excitation at the absorption maximum (430 nm). Figure 1 inset compares the two emission spectra. As is shown, when the sample is excited at 360 nm the resulting emission spectrum encompasses the smaller peak at 475 nm, leading to only one distinct peak in the emission spectrum. The spectral variations suggest that two different species of BTA-2 are present in the solution. To ensure that the spectral shifts were not due to impurities from the synthesis; absorption, emission, and excitation spectra were collected for the two starting materials: benzothiazole and N,N-dimethylaniline. The starting materials only had spectra that were in the UV region and did not overlap with any of the BTA-2 spectra in solution (data not shown). Thus, the spectral shifts in the BTA-2 solutions were not the result of impurities in the solution but resulted from two distinct forms of the BTA-2 in the solution. One species is highly absorbent at 430 nm, but not particularly fluorescent; while the other is more fluorescent and dominates the emission at 475 nm. The likely cause of the 430 nm peak is the formation of BTA-2 aggregates or micelles in the solution. These are expected to dominate the concentration and resulting absorbance. However, the highly localized dye concentration results in a substantially decreased fluorescent quantum yield. ThT has been found to form micelles in solution [27] and since BTA-2 has a similar structure, but not positively charged, it is more likely to form micelles in aqueous solutions.

Anisotropy measurements were performed to determine what type of molecular species the 430 nm absorption peak was and if it was in fact the result of micelle formation. If

so, its emission will show an anisotropy significantly larger than zero, as large micelle species exhibit no appreciable rotation during the fluorescence lifetime. If the anisotropy is zero, then the emission must result from a species that is rotating on a time scale that is fast compared to the excited state lifetime. The fluorescence anisotropy was measured for BTA-2 in pH 2 water by exciting at 420 nm, and was found to have an average anisotropy of 0.28. When the same sample was excited at 370 nm, the average anisotropy was calculated to be 0.06. The anisotropy measurements indicate that two different species are present in the solution. The slower rotating BTA-2 micelles absorb at 430 nm and emit at 475 nm. While the faster rotating fully solvated BTA-2 molecules absorb at 370 nm and emit at 440 nm. The absorption spectrum is dominated by the high concentration, low quantum yield micelles, while the fluorescence spectrum is dominated by the lower concentration fully solvated BTA-2 molecules.

Since BTA-2 formed micelles in a 2% CH_3CN solution, it was hypothesized that increasing the CH_3CN concentration would solvate the BTA-2 and reduce the micelle concentration. Therefore, a solution containing BTA-2 in a 1:1 mixture of water and CH_3CN was made to investigate how increasing the CH_3CN concentration affects the micelle formation. Figure 2 shows the normalized absorption, emission, and excitation spectra acquired for BTA-2 in a solution diluted with a 1:1 mixture of CH_3CN and water. The absorption spectrum of BTA-2 in the 1:1 mixture had a larger peak at 365 nm and a small shoulder at 430 nm. The 430 nm peak corresponds to the peak observed in the absorption spectrum of BTA-2 in pH 2 water. When the sample was excited at 365 nm, the emission spectrum yielded a single peak centered at 440 nm. The excitation spectrum revealed a peak at 385 nm and a very small peak at 430 nm that can only be observed when the region is enlarged. When the sample was excited at 430 nm, a peak at 475 nm was observed, which is similar to what was observed in the BTA-2 in pH 2 water sample. However,

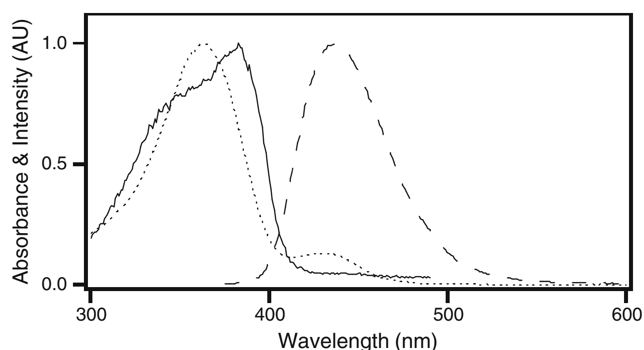


Fig. 2 BTA-2 in a 1:1 mixture of CH_3CN and water. B) Normalized absorption spectrum (*dotted line*), excitation spectrum (*solid line*), and emission spectrum (*dashed line*)

when the two emission spectra were compared, the 440 nm emission peak (free BTA-2) was 80x more intense than the 475 nm peak (micelles). If the solution was diluted more, then the absorption peak at 430 nm disappears, leaving a single peak in the absorption spectrum at 365 nm. The disappearance of the red-shifted peak at 430 nm indicates that the micelles present in the solution dissociate when diluted. Small molecule dye aggregates are known to have red-shifted absorption peaks that upon dilution can be dispersed, leading to the disappearance of the absorbance associated with the micelles [43]. Fluorescence anisotropy measurements were taken for both peaks in the excitation spectrum. When the sample of BTA-2 in 1:1 mixture of water and CH_3CN was excited at 420 nm, the average anisotropy was calculated to be 0.22, indicating that the slowly rotating micelles were present. When anisotropy measurements of free BTA-2 were taken with 365 nm excitation, the average anisotropy was found to be 0.011. The higher anisotropy with 420 nm excitation indicates that even solutions with a higher CH_3CN concentration some micelles remain.

Micelles formed in both BTA-2 solutions containing either 2% or 50% CH_3CN resulted in a red shifted peak in the absorption and excitation spectra. When the different solutions were compared, similar spectral behavior was observed in both cases; however, the absorption spectrum of BTA-2 in pH 2 water had only a single absorption peak at 430 nm. This suggests, the majority of the BTA-2 molecules exist as micelles and that the fully solvated BTA-2 is below the detection limit of the instrument. The fluorescence spectra reveal the micelles to be much weaker in fluorescence and thus the small free dye dominates the emission spectra. The anisotropy measurements confirm the assignment of the 430 nm absorption to micelles, given it has an anisotropy that is significantly larger than zero. Conversely, the near zero anisotropy shows the absorbance at 365 nm to be free dye in solution. In the 1:1 mixture of water and CH_3CN, the majority of BTA-2 was found to be freely solvated; however, there was still a small micelle peak present suggesting that even 50% CH_3CN cannot fully solvate all the BTA-2 micelles. Experiments in pure CH_3CN, show that only fully solvated dye is present in solution. The absorption spectrum of BTA-2 in pure CH_3CN showed no red shifted peak, even at high concentrations of BTA-2, and had an average anisotropy of 0.004 (data not shown). BTA-2 micelles disperse upon addition of CH_3CN leaving only monomer dye present in solution and a single absorption peak at 360 nm. The decrease in micelle formation between samples was confirmed by the anisotropy measurements (data not shown). There was a small decrease from 0.28 to 0.22 in the anisotropy at the micelle emission (475 nm) at the higher CH_3CN concentration. These results suggest that

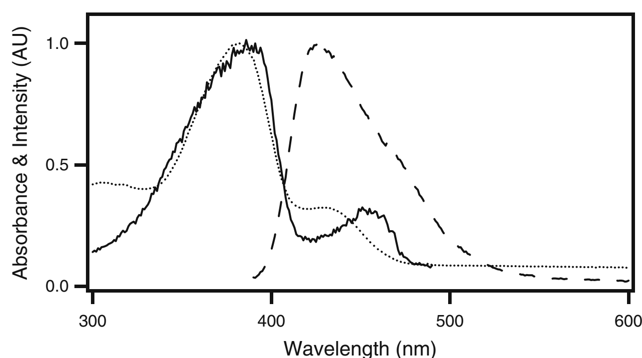


Fig. 3 BTA-2 bound to fibrils in pH 2 water. Absorption (*dotted line*), excitation (*solid line*), and emission (*dashed line*) spectra

water promotes the formation of BTA-2 micelles while CH_3CN favors the free monomeric BTA-2.

With an understanding of the photophysics of BTA-2 in solution, the properties of the dye in the presence of amyloid fibrils were explored. Figure 3 shows the normalized spectra for BTA-2 in the presence of fibrils in pH 2 water (2% CH_3CN) with a BTA-2 concentration of 62.90 μM. The absorption spectrum shows two distinct peaks at 380 nm (larger) and 430 nm (smaller). The 430 nm peak was the same feature observed in solution and is attributed to micelles. Presumably the 380 nm peak corresponds to dye bound to the amyloid fibrils. An emission spectrum was acquired by exciting at 380 nm and showed a maximum emission at ~430 nm, which is blue-shifted 20 nm from the emission peak of free BTA-2 in the same solution. The excitation spectrum for the emission at 500 nm had a maximum peak at 380 nm with a smaller peak at 450 nm. Unlike the free dye in pH 2 water, the dye with fibrils yields the same maximum (380 nm) in both absorption and excitation spectra. Thus, the predominant species in solution is now the most fluorescent. When the sample was excited at the 430 nm peak (micelle peak), an emission spectrum was obtained with a maximum at 475 nm and a 14 fold decrease in intensity relative to the emission spectra taken when excited at 380 nm (bound BTA-2). Fluorescence anisotropy measurements were acquired to confirm these assignments and determine if the spectral shifts observed were due to BTA-2 binding to fibrils. The anisotropy of the emission at 430 nm generated by 380 nm excitation was found to be 0.33. This large value indicates that the dye is rotating very slowly, as expected for the dye bound to the large fibrils. Given the size of the fibrils one might expect the anisotropy to be at its maximum value of 0.40, indicating no rotation between absorption and emission. However, it is not clear if the absorption and emission dipoles for BTA-2 are exactly parallel. Assuming no rotation during the excited state lifetime, the measured anisotropy yields an angle of 20 degrees between the absorption and emission transition dipoles for BTA-2.

Although it was determined that BTA-2 in a pH 2 solution, mostly exists as micelles, it was determined that binding still occurred. Figure 4 shows a comparison of the normalized excitation and emission spectra of BTA-2 free and bound to fibrils in pH 2 water. When BTA-2 is free in solution, the excitation maximum is at 370 nm and the emission maximum is at 450 nm; upon binding to the fibrils the excitation maximum red-shifts to 380 nm and the emission maximum blue-shifts to 430 nm. There is also an increase in the total fluorescence intensity by a factor of 27. This trend is similar to what is observed when ThT binds to amyloid fibrils [23, 25, 27, 29, 31]. The BTA-2 acts as a molecular rotor when free in solution, then when it is confined in the fibrils, the BTA-2 planarizes, which accounts for the shifts observed in the excitation and emission spectra along with the increase in fluorescence intensity [31, 44, 45].

The binding of BTA-2 to amyloid fibrils in a 1:1 mixture of CH_3CN and water was investigated to determine how the incorporation of CH_3CN affects binding. It was previously shown that additional CH_3CN decreased the amount of micelles present in solution. Absorption, excitation, and emission spectra were taken of the BTA-2 in the presence of fibrils in a solution diluted with a 1:1 mixture of CH_3CN and water. The absorption spectrum shows a maximum at 380 nm with a very small peak at 430 nm. While the excitation spectrum for the emission at 500 nm had a peak at ~385 nm, which is similar to the excitation of the free BTA-2 in the same solution. The emission spectrum acquired with 365 nm excitation shows a peak at 440 nm, and has an overall intensity very similar to that of the free BTA-2. When the free BTA-2 spectra in the 1:1 mixture of water and CH_3CN was compared to the BTA-2 spectra when fibrils were present (Fig. 5), there was no spectral shift observed between the two solutions. This indicates that BTA-2 is not binding to the amyloid fibrils under these conditions. The fluorescence anisotropy was measured to determine if the binding could be detected using a previously validated method. The anisotropy for 380 nm

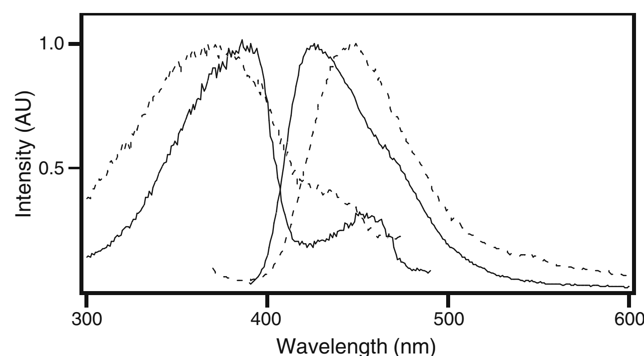


Fig. 4 Excitation and emission spectra of free BTA-2 (*dashed lines*) and bound to fibrils (*solid lines*) in pH 2 water

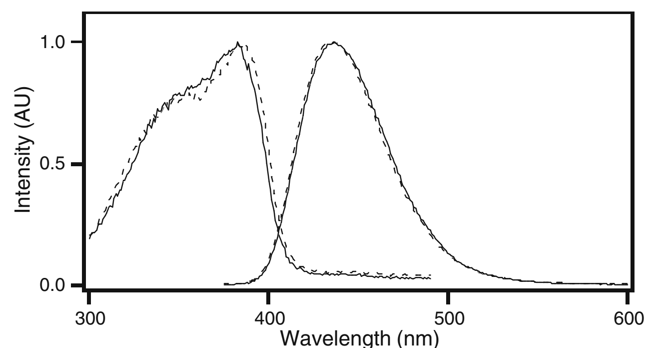


Fig. 5 Excitation and emission spectra of free BTA-2 (*solid lines*) and bound to fibrils (*dashed lines*) in 1:1 mixture of water and CH_3CN

excitation was found to be only 0.03, indicative of a freely rotating dye molecule; therefore in a solution with a high CH_3CN concentration there exists competition for binding of the BTA-2 to the fibrils and solvation in the CH_3CN. Presumably this would be the case with nearly any other solvent, such as DMSO, used to aid the solvation of the BTA-2 in water. As such, great caution should be used in such experiments to minimize the concentration of the second solvent to prevent competition for the hydrophobic dye between amyloid binding and solvation in the less polar solvent.

To further study this effect, a series of experiments examined the binding of the BTA-2 to amyloid fibrils as function of CH_3CN concentration. A series of solutions containing only BTA-2 and BTA-2 with fibrils were made containing varying concentrations of CH_3CN (from 2%-50% CH_3CN). Absorption, emission, and excitation spectra were taken for all solutions. Anisotropy measurements were only collected for the solutions containing fibrils. When the solution contained only BTA-2, the absorption maximum at 430 nm (micelles) increased when the amount of CH_3CN was between 2% and 25% (Fig. 6A). At 25% CH_3CN a peak at 365 nm started to form and upon increasing the CH_3CN concentration even further, the 365 nm peak (free BTA-2) became the dominant peak, while the 430 nm peak (micelles) remained relatively constant. When BTA-2 is in pure CH_3CN, no absorption peak at 430 nm is observed. The emission spectra of the same solutions excited at 365 nm (absorption of the free BTA-2) revealed only one peak that increased in intensity with the increasing CH_3CN concentration. This suggests that by increasing the amount of CH_3CN in solution, the CH_3CN solvates the BTA-2 micelles, leading to an increase in fluorescence and observation of a new absorption peak at 360 nm. When fibrils are introduced into a solution of BTA-2 and 2% CH_3CN, the absorption spectrum had two peaks, one at 430 nm (micelles) and the other at 380 nm (bound). As the percentage of CH_3CN was increased the bound peak decreased, while the micelle peak increased slightly. When the concentration of CH_3CN was increased past 25%, the absorption peak at 380 nm blue shifted to 365 nm (solvated BTA-2) with an increase in absorbance. The emission spectra of the same fibril solutions revealed a red-shift in the emission that increased in intensity. The spectral changes observed in the absorption and emission spectra indicate that increasing the amount of CH_3CN prevented binding to the fibrils and leads to free dye in solution. To further confirm this hypothesis, anisotropy measurements were taken for each fibril solution using 380 nm excitation (wavelength were BTA-2 bound absorbs). A plot of anisotropy as a function of CH_3CN concentration shows that as the concentration increases, the anisotropy decreases (Fig. 6B). This confirms what was observed in the absorption and emission spectra, and it is clear that increasing the amount of CH_3CN in the solution increases the BTA-2′s solubility, and hinders binding to the amyloid fibrils. Since BTA-2 is not water soluble, the CH_3CN is necessary to keep the BTA-2 solvated in aqueous solutions, but when the amount CH_3CN is increased beyond 25%, the CH_3CN preferentially solvates the BTA-2 dye and hinders binding. Thus in the solution containing 50% CH_3CN, the

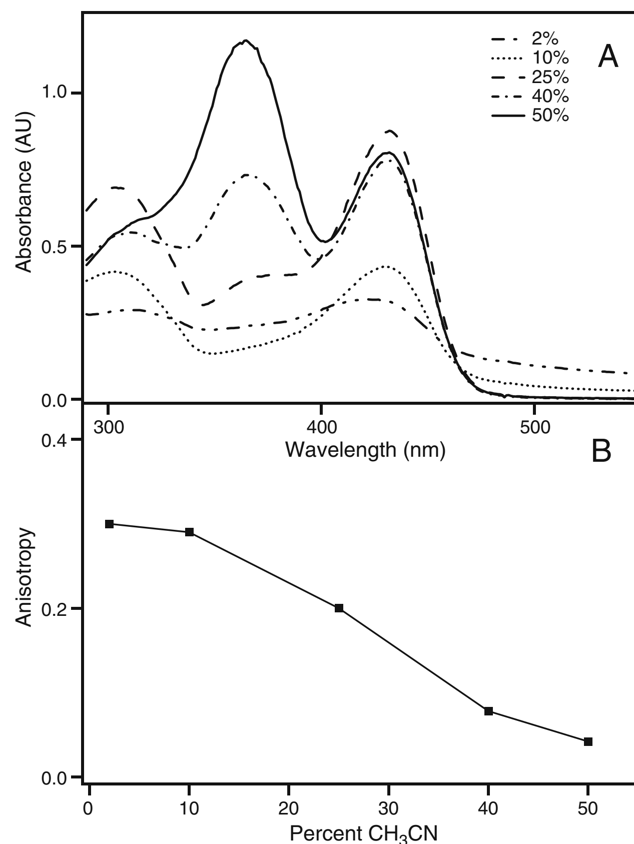


Fig. 6 **a** Absorption spectra of BTA-2 with different amounts of CH_3CN present. **b** Anisotropies of BTA-2 with fibrils versus the amount of CH_3CN present in solution

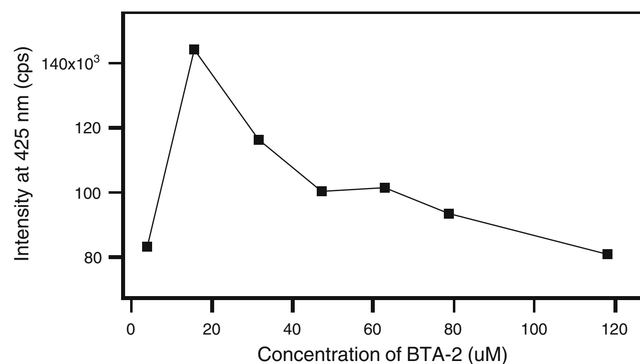


Fig. 7 Emission intensity at 425 nm versus the concentration of BTA-2 present in solutions containing fibrils

BTA-2 and fibrils are actually isolated from one another and the CH_3CN prevents binding.

Since the amount of CH_3CN affects the binding to the fibrils, a series of experiments were performed to determine if the amount of BTA-2 present in solution also impacted binding. A series of solutions containing BTA-2 with and without fibrils at different concentrations, from 4 uM to 118 uM, were prepared (note: the amount of CH_3CN present in all the solutions was 2%). In the solutions containing only BTA-2, the absorption, emission, and excitation spectra all increased in intensity with increasing BTA-2 concentration, as one would expect. As the BTA-2 concentration was increased, an increase in scattering from the micelles in the absorption spectra was also observed. For solutions that contained fibrils, the absorption spectra showed an increase in the peaks at 380 nm (bound BTA-2) and 430 nm (micelles) with increasing BTA-2 concentration, but the emission spectra showed a different effect. Figure 7 shows the intensity of the emission spectra at 425 nm as the concentration of BTA-2 present in the solutions containing fibrils is varied. The graph shows that there is a maximum concentration of 15.7 μM that yields the greatest fluorescence from the bound BTA-2; below or above this concentration the fluorescence intensity decreases. This trend indicates that the fibrils in solution have a fixed number of binding sites available for BTA-2 and at low concentrations there are more binding sites available than BTA-2 molecules. As more BTA-2 molecules are added, the fluorescence intensity increases until the available binding sites have saturated which is when the fluorescence maximum is reached. Subsequently, as more BTA-2 dye is added after the binding sites are occupied, there is a decrease in fluorescence intensity attributed to the self-quenching from the excess dye.

ThT is one of the most common fluorescent dye currently used to label or probe amyloid fibrils. BTA-2 and ThT are structurally similar dyes that both exhibit similar spectral properties when bound to fibrils and free in solution; therefore, it is important to directly compare these two dyes in order to understand how the spectral properties differ and determine if one dye is better suited for fibril studies. Two solutions were prepared for each dye, one with the free dye only and another that contained fibrils and dye. All the solutions contained 2% CH_3CN in pH 2 water. Each solution was excited at its absorption or excitation maximum: free ThT was excited at 415 nm, ThT bound to fibrils was excited at 440 nm, free BTA-2 was excited at 360 nm, and BTA-2 bound to fibrils was excited at 380 nm. Figure 8 shows the emission spectra for all four solutions. Neither of the two dyes is particularly fluorescent when free in solution; however, BTA-2 was found to be 3.6 times more fluorescent than ThT. When the spectra of bound ThT and bound BTA-2 were compared, the bound BTA-2 emission was 2.3 times larger than the bound ThT; the significant difference in fluorescence intensity between the two dyes suggests that BTA-2 is a better dye for spectroscopic studies of amyloid fibrils.

Conclusions

BTA-2 has distinct absorption and emission characteristics in solution and when bound to amyloid fibrils, which makes it an interesting dye for use in identifying amyloid fibrils using spectroscopy. In aqueous solution, it was determined that BTA-2 forms micelles. These micelles exhibit a red-shifted absorption from the freely solvated dye and have a significantly lower quantum yield than the monomer dye. The presence of the micelles was confirmed with fluorescence anisotropy experiments and from the fact the micelles could be dispersed with the addition of CH_3CN in solution. Upon increasing the CH_3CN concentration, the absorption spectrum showed a decrease in the micelle peak and the appearance of a new monomer peak that previously could only be observed in the excitation spectrum. While

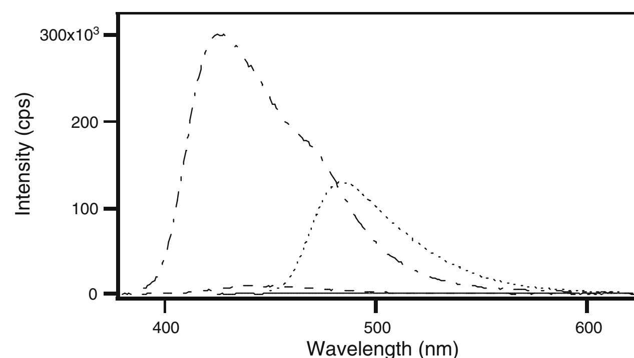


Fig. 8 Comparison the fluorescence of ThT to BTA-2 free and bound. Solid line is the fluorescence of free ThT, dotted line is the fluorescence of ThT bound to fibrils, the dashed line is the fluorescence of free BTA-2, and the dashed-dotted line is the fluorescence of the BTA-2 bound to fibrils

the addition of the CH_3CN helped to fully solvate the dye, its addition hindered the binding of the dye to the amyloid fibrils, whereas the presence of the micelles did not. BTA-2 was determined to bind to amyloid fibrils in aqueous solutions containing less than 25% CH_3CN; above this amount binding did not occur. In practice, the inclusion of a second solvent to bring the dye into aqueous solution should be minimized to avoid competition for the dye binding to the fibrils. It was also determined that the concentration of BTA-2 present in fibril solutions affected the fluorescence intensity, suggesting that above a specific concentration excess BTA-2 quenches the bound BTA-2 fluorescence. Finally, in comparing BTA-2 to the well studied ThT, it was found that BTA-2 is a much more fluorescent dye than ThT, free and when bound to amyloid fibrils. In this paper we have characterized BTA-2 binding to amyloid fibrils in solution and demonstrated it is an excellent new dye for studying amyloid fibrils with spectroscopy.

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