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Amphotericin B interactions with soluble oligomers of amyloid Aβ1-42 peptide

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ABSTRACT

Amphotericin B has recently been suggested as an efficient inhibitor of amyloid peptide fibril formation; however its interactions with more neurotoxic, soluble forms of amyloid peptides have not been reported to date. Circular dichroism spectroscopy allowed for distinguishing between the binding and inhibition of aggregation events: amphotericin B distinctly interacts with both unordered and ordered, β-structure-rich soluble oligomeric forms of Aβ1-42 peptide, yet amphotericin B has no measurable impact neither on the secondary structure nor on time-dependent aggregation profile of the amyloid peptide.

1. Introduction

Amyloid peptides (Aβ), which are composed of 40-42 amino acid sequences, for example, Aβ1-40 and Aβ1-42, have been implicated as the main suspects that are responsible for the occurrence and progression of Alzheimer's disease.[1] In particular, conformational changes from unordered to structured, β-sheet-rich soluble, pre-fibrillar oligomers of Aβ1-42 have been suggested as the key neurotoxic events.[2–4] Currently, the recognition of soluble oligomers of amyloid peptides presents a challenge.

In view of accessibility and ease of structural and functional modification, small molecules that can bind to soluble oligomeric Aβ1-42 species are of immense interest not only as potential leads for therapeutic intervention of Alzheimer's disease, but also as useful motifs for studying conformational changes and aggregation behavior of the amyloid peptides.

Several recent accounts indicated that amphotericin B, AmB (Fig. 1), a polyene macrolide antibiotic can inhibit the formation of fibril-like species of Aβ25-35[5] and Aβ1-40[6,7] amyloid peptides. However, the ability of AmB to affect the conformational changes of neurotoxic soluble oligomers of amyloid peptides, in particular Aβ1-42 peptide, has not been reported thus far. Notably, AmB was also shown to modulate the aggregation process of prion protein,[8] albeit no mechanistic details have been provided. Therefore, understanding the molecular mode of AmB's action is of interest and significance in relation to several protein-misfolding diseases.

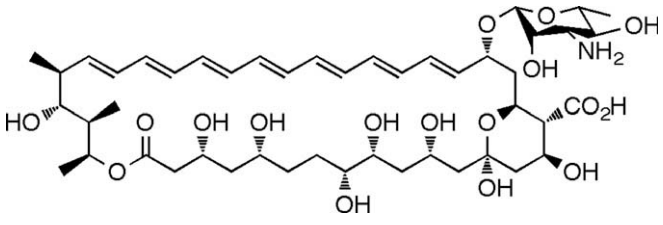

Figure 1. Structure of amphotericin B, AmB.

Aggregation of amyloid peptides is a complex phenomenon. Spectroscopic tools have been used extensively to investigate key events and species responsible for the formation of Aβ-aggregates. In particular, circular dichroism (CD) has been an invaluable spectroscopic tool for assessing the conformational transitions of amyloid species.[9,10] The advantage of CD over many other spectroscopic and imaging techniques, including dye-binding methods, is that CD allows for monitoring conformational transitions of soluble, pre-fibrillar oligomers of amyloid peptides. CD has also been used to gain insight into the effect of various small molecules on amyloid peptides aggregation.[11–14] Importantly, when a small molecule is chiral (and can undergo a chiroptical change upon interaction with other species), one can monitor changes that are occurring in both the chromophore and in the peptide spectral regions upon their mutual interaction. This is significant, since an affinity of a compound towards amyloid peptides might not necessarily indicate that the compound is an inhibitor of an amyloid-aggregation process. Compounds that can recognize amyloid aggregates, can potentially provide an insight into the structural aspects of amyloid species, which should be beneficial for the design of efficient inhibitors of the amyloid aggregation.

Aβ self-aggregation is a dynamic, highly heterogeneous process and an evaluation of the impact of a small molecule on distinct

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conformations of amyloid peptide presents a challenge. The inhibitory effect of AmB on the Aβ-aggregation process, which led to the fibril formation, had been established by Congo red[5] and thioflavin T[6,7] dye-binding assays. These dye-binding assays are widely and routinely used for assessing the aggregation profile of amyloid peptides as well as for estimating the anti-aggregation potential of various small molecules.[15] One of the main limitations of these dye-based assays, however, is that the dyes are binding exclusively to fibrillar aggregates, whereas no spectral changes are observed in the presence of soluble oligomeric peptides.[15] Thus, these assays are not suitable for detecting conformational changes that are occurring in the soluble neurotoxic oligomeric amyloid species. In addition, a few literature accounts reported that the dye-binding assays might be prone to false positive effects.[16,17] This can be rationalized by the notion that both the inhibitor's and the dye's molecules might compete for the same binding site(s) of the amyloid aggregates; therefore, a small molecule that simply binds to amyloid aggregates can be mistaken for an inhibitor. Another limitation of the dye-binding assays is that the conditions that are used for performing accurate and reproducible Congo red and thioflavin T assays often differ from amyloid-aggregation assays, which might disrupt the amyloid self-assembly.[18] In this light, techniques that are based on non-dye binding approaches for monitoring amyloid—small molecule interactions should aid in an unambiguous evaluation of anti-aggregation potential of small molecules. Hence, chiroptical spectroscopy becomes particularly important for evaluating the ability of small molecules to interact with soluble oligomers of amyloid peptides.[11]

Here, we report on the interaction of distinct secondary structure conformations of soluble oligomeric Aβ1-42 peptide with AmB using CD spectroscopy. Our results demonstrate that although AmB has a measurable affinity towards both unordered and ordered soluble oligomers of Aβ1-42, it does not modulate neither the secondary structure nor time-dependent aggregation behavior of the amyloid peptide. Although our results indicate that the ability of AmB to act as an inhibitor of Aβ-aggregation process is doubtful, AmB might serve as a molecular spectroscopic probe for various oligomeric forms of Aβ1-42 peptide.

2. Results and discussion

In the present study, we used CD spectroscopy to characterize AmB self-association, estimate the secondary structure conformations of Aβ1-42 oligomers as well as to probe the binding of AmB monomer to soluble oligomers of Aβ1-42.

The conformation and kinetics of Aβ peptide aggregation strongly depends on the composition and pH of the medium as well as on the peptide concentration. Hence we investigated these parameters to achieve the optimum set of conditions.

Low solubility of many organic compounds, including AmB, in aqueous buffers always requires the addition of organic solvents in order to study the effect of small molecules on the conformational transitions of amyloid peptides. This imposes some restrictions on the type of solvents that can be used for in vitro assays. It should be noted that organic solvents have also been known to alter the aggregation behavior of amyloid peptides,[11] and therefore, their amounts should be carefully controlled. DMSO and DMF are the only solvents capable of solubilizing AmB, however, they are not transparent to far-UV light. Therefore, we kept the concentration of DMSO to a minimum in all experiments to allow for monitoring of the Aβ1-42 peptide conformational transitions in the far-UV region along with assessing the chiroptical responses of AmB.

TRIS buffers are one of the most suitable media for assessing protein conformation using CD,[19] hence it was utilized here. We also found that basic medium (pH 8.7) was absolutely required to obtain a reproducible random coil to β-sheet transition of Aβ1-42 soluble oligomers. The need for high pH media is consistent with literature accounts,[11] as we were unable to obtain a random coil conformation of Aβ1-42 at pH lower than 8.0.

With respect to concentration, we found that 25 μM Aβ1-42 exhibited a conformational transition from unordered to ordered oligomers within several days while still yielding soluble amyloid species. Importantly, independent preparations of 25 μM Aβ1-42 were reproducible with respect to the initial and final conformation of the peptide as well as to the kinetics of the conformational transition. On the other hand, concentrations below 25 μM prolonged the conformational transition from unordered to β-sheet Aβ1-42 species to several weeks. In addition, signal-to-noise ratios in CD spectra required long accumulation times, which should have been avoided due to the light-sensitive nature of AmB. Higher concentrations of Aβ1-42 (above 25 μM) tended to promote rapid aggregation of the peptide, while leading to a formation of insoluble aggregates. Thus, 25 μM Aβ1-42 concentration was utilized throughout. It was found that the final amount of DMSO (0.015% v/v) did not have a measurable effect neither on the initial conformation nor on the time-dependent aggregation kinetics of Aβ1-42 as established by CD measurements.

Under the described above conditions, freshly prepared 25 μM Aβ1-42, exhibited a CD spectrum indicative of an unordered oligomeric species with a characteristic minimum at ca. 200 nm (Fig. 2A). A transition to ordered β-structure-rich species, with a characteristic minimum at 216 nm, was observed within two days (Fig. 2A); no significant changes in the CD spectra were occurring after 48 h of incubation and up to 72 h. An isosbestic point at *ca.* 207 nm was also observed, thus confirming a two-species conformational transition. This aggregation process was also monitored by light scattering and showed a good correlation with CD data (Fig. 2B and C): a steady increase of both scattering intensity and hydrodynamic radius was observed within 72 h. Notably, no precipitation of the peptide was observed during this period of time. Collectively, these data suggested that Aβ1-42 underwent a transition from unordered to β-sheet-like soluble oligomeric species.

In order to evaluate the affinity of AmB towards soluble Aβ oligomers, we first examined the self-association of AmB by using absorbance and CD spectroscopy. The changes in the absorbance spectra of AmB proved to be fairly complex and not substantial to allow for an unambiguous determination of an equilibrium constant. Hence, the self-association of AmB was determined exclusively by CD spectroscopy. Furthermore, with the aim to establish the effect of AmB on the conformational transitions of Aβ1-42, we avoided the use of a water-soluble formulation of AmB, that is, Fungizone®. One of the components of this formulation is sodium deoxycholate, and steroids have been shown to affect the aggregation of amyloid peptides.[20–22] The presence of such a component in the mixture might obscure the evaluation of AmB-amyloid interaction.

AmB exhibited an asymmetric couplet in the CD spectrum with 328 nm maximum and 350 nm minimum (Fig. 3), which is indicative of dimeric or oligomeric species.[23–25]

It was reported that monomeric AmB does not exhibit any CD transitions in the 300–400 nm range.[24,25] Consistent with this observation, we did not observe any CD of AmB in trifluoroethanol (data not shown), a solvent which is known for its anti-aggregation ability. The dependence of the CD signals, θ_{328} and θ_{350} as a function of AmB total concentration, [AmB], is not linear. This behavior is also consistent with the documented AmB self-association phenomenon.[23–25]

For simplicity, based on literature accounts, we assumed that self-association of AmB follows a monomer-dimer equilibrium.[26,27] Thus, the dimerization constant, α, can then be expressed by Eq. 1:

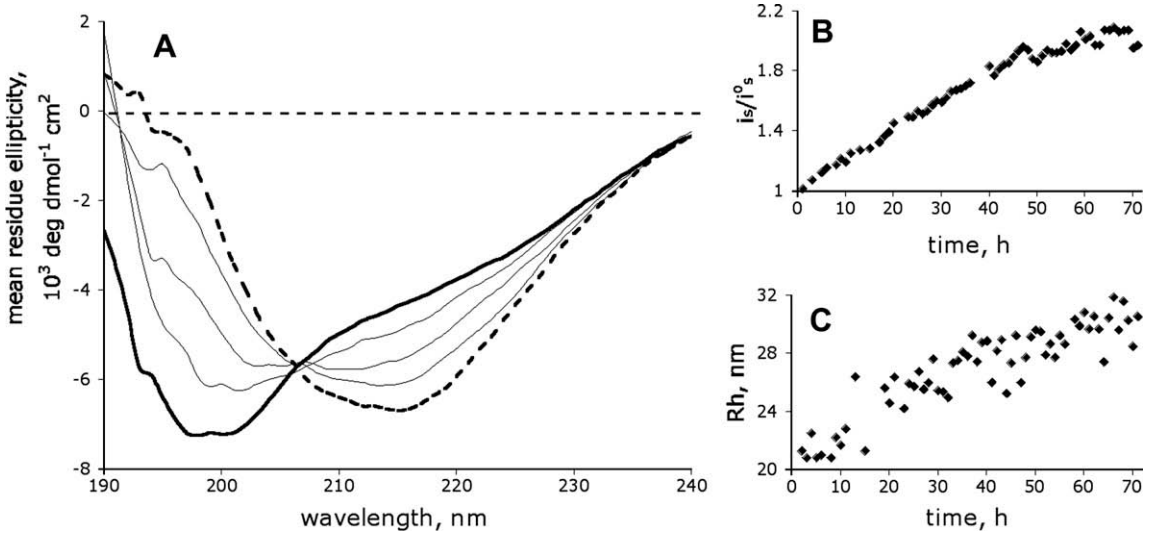

Figure 2. Representative time-dependent aggregation CD spectra of Aβ1-42 (**A**) from 0 h (bold line) to 72 h (dashed line); time-dependent dynamic light scattering data: scattered light intensity (**B**), and hydrodynamic radius (**C**); in 10 mM TRIS/NH_4OH buffer (pH 8.7).

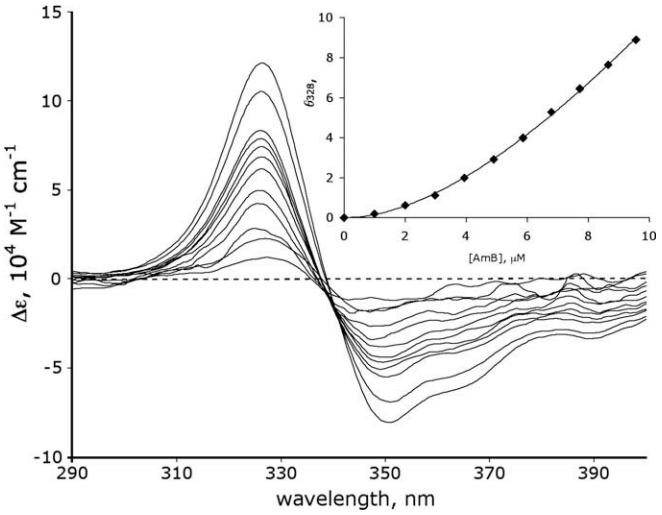

Figure 3. Representative CD spectra of AmB titration 1.00–13.09 μM into 10 mM Tris/NH_4OH (pH 8.7) buffer. Inset: CD intensity at the 328 nm maximum as a function of AmB concentration; the data are the average of two independent titrations.

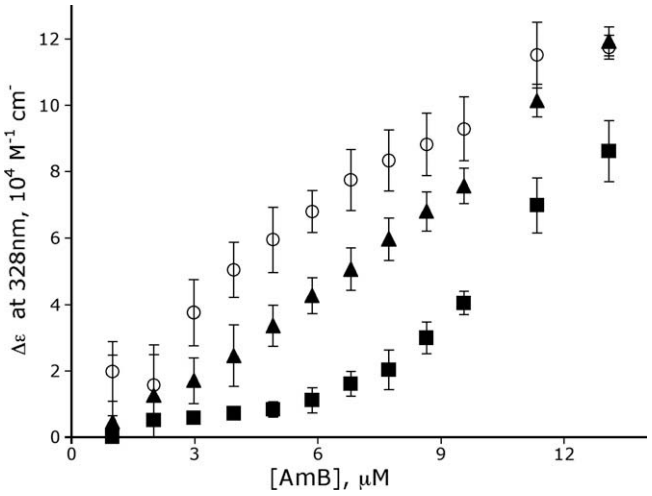

Figure 4. Molecular ellipticity of AmB in the absence of Aβ1-42 (○), in the presence of unordered oligomeric Aβ1-42 (▲), and in the presence of β-sheet oligomeric Aβ1-42 (■), in 10 mM TRIS/NH_4OH buffer (pH 8.7). Data are the average of two independent preparations of Aβ1-42 ± SD. See Section 4 for details.

$$\alpha = [D]/[M]^2 \quad (1)$$

where M and D denote monomeric and dimeric species of AmB, respectively. Since M is silent in CD, the AmB total concentration can be related to the CD signal, according to Eq. 2:

$$[AmB] = [(\theta/\Delta\varepsilon_D)/\alpha]^{1/2} + 2(\theta/\Delta\varepsilon_D) \quad (2)$$

where [AmB] was determined from the mass balance [AmB] = [M] + 2[D], and $\Delta\varepsilon_D$ is the dimer's extinction coefficient. Using non-linear least square method, we determined $\alpha = 0.021 \pm 0.004$ and $\Delta\varepsilon_D = 8.0 \times 10^5\ M^{-1}\ cm^{-1}$ at 328 nm and $-5.0 \times 10^5\ M^{-1}\ cm^{-1}$ at 350 nm. We found that AmB does not abide by the dimerization model at the concentrations above 10 μM, and, therefore, our quantitative analysis was restricted to this concentration range. By varying the fitted concentration interval (Fig. 3, inset) no change in the value of α was observed for [AmB] lower than 10 μM.

Next, AmB was titrated into the solution of unordered conformation of 25 μM Aβ1-42 amyloid peptide (Fig. 4). The CD intensity of AmB in the presence of the Aβ1-42 was appreciably lower than the intensity of AmB in the absence of Aβ1-42, which suggested that AmB was binding to these soluble unordered aggregates. Titration of AmB into the aggregated, β-sheet-rich, yet still soluble, oligomeric Aβ1-42 had led to even more pronounced changes in the molecular ellipticity of AmB (Fig. 4). Importantly, CD spectra in the far-UV region revealed that 13 μM AmB did not have any measurable effect on neither the initial conformation nor the time-dependent aggregation profile (up to 24 h) of 25 μM Aβ1-42 peptide, which indicated AmB is not acting as an inhibitor of Aβ1-42 aggregation process.

In order to explain the observation that titration of AmB solution into 25 μM solution of Aβ1-42, either in its unordered or β-structure oligomeric forms, led to a distinct and substantial decrease of the dimeric AmB (Fig. 4), we considered two possible scenarios: (i) the binding constant of AmB to the β-sheet oligomers of Aβ1-42, K_β, is higher than the binding constant of AmB to the unordered, random coil conformation of Aβ1-42, K_{rc}, or (ii) the number of soluble aggregates in the ordered, β-sheet-rich conformation, n_β,

has increased, thus providing more binding sites for AmB as compared to the number of soluble aggregates in the unordered, random coil conformation of Aβ1-42, n_{rc}.

Based on the literature accounts,[28,29] we assumed that only M is binding to the amyloid peptide. In this case, the mass balance becomes [AmB] = [M] + 2[D] + v[Aβ1-42], where v is the number of M molecules bound to Aβ1-42.

This allowed us to apply the Schatchard model to the binding of monomeric AmB to Aβ1-42, as shown in Eq. 3:

$$v/n = K[\mathrm{M}]/(1 + K[\mathrm{M}]) \quad (3)$$

where n is the number of sites per Aβ1-42 molecule and K is the binding constant. Since the intensity of the observed CD signal is related to the amount of D, that is, dimeric, unbound AmB, we can use the mass balance combined with Eqs. (1)–(3) to calculate [AmB] as shown in Eq. 4:

$$[\mathrm{AmB}] = [(\theta/\Delta\varepsilon_{\mathrm{D}})/\alpha]^{1/2} + 2(\theta/\Delta\varepsilon_{\mathrm{D}}) + n[\mathrm{A}\beta 1-42] \times K[(\theta/\Delta\varepsilon_{\mathrm{D}})/\alpha]^{1/2}/(1 + K[(\theta/\Delta\varepsilon_{\mathrm{D}})/\alpha])^{1/2} \quad (4)$$

The experimental data, which was obtained from CD titrations of AmB into the Aβ1-42 solutions, using both θ_{328} and θ_{350} values from two independent preparations of Aβ1-42, were fitted into Eq. 4 using non-linear least square method (Fig. 5). Plotting the concentration of the dimeric AmB, as a function of the total concentration of AmB, allowed for the calculation of K and n for both the random coil and β-sheet-rich oligomers of Aβ1-42.

Obtained binding constant values $K_{rc} = 0.32 \pm 0.22\ \mu M^{-1}$ and $K_{\beta} = 0.49 \pm 0.13\ \mu M^{-1}$ indicate that the affinity of AmB towards both forms of soluble Aβ oligomers is virtually identical. This suggests that the number of binding sites is responsible for the shift of AmB's monomer-dimer equilibrium towards the monomeric AmB. This is supported by a ca. threefold increase in n, that is, $n_{\beta} = 0.245 \pm 0.026$ as compared to $n_{rc} = 0.085 \pm 0.030$. Since the peptide is undergoing an aggregation process, it is reasonable that the number of potential binding sites would increase. Tentatively, we can propose that in a random coil conformation it requires a multiple of ca. 11 peptide molecules to create a binding site for AmB, and in a β-sheet conformation of Aβ1-42 it takes a multiple of ca. 4 Aβ1-42 molecules. However, the binding affinity of both forms of Aβ-aggregates towards AmB is similar in both cases. It should also be pointed out that due to the dynamic nature and not well-defined structure of Aβ-oligomers, the term *binding site* is used here for illustrative purposes only.

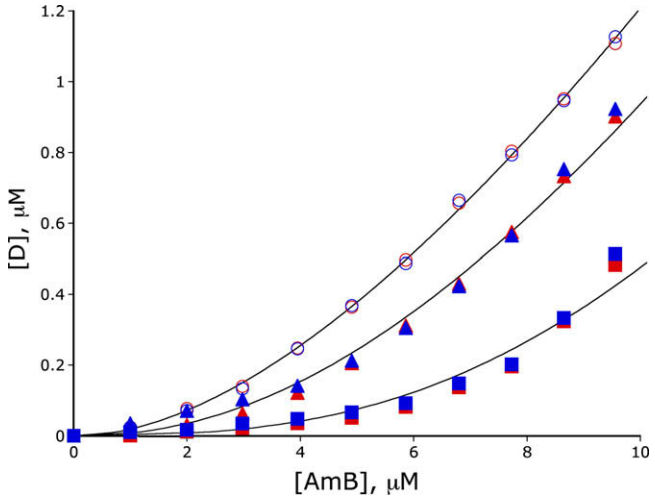

Figure 5. Calculated fits of the concentration of AmB dimer as a function of total AmB concentration, [AmB]. Data are the average of two independent preparations of Aβ1-42 using θ_{328} (red symbols) and θ_{350} (blue symbols) values, in the absence of Aβ1-42 (○), in the presence of unordered oligomeric Aβ1-42 (▲), in the presence of β-sheet oligomeric Aβ1-42 (■).

As pointed out earlier, AmB had no measurable impact neither on the conformations of Aβ1-42 nor on the kinetics of Aβ1-42 aggregation. Thus, due to M(CD silent) ⇆ D(CD active) equilibrium, that is affected by Aβ1-42 species, AmB might serve as a potential spectroscopic probe for estimating the nature of soluble Aβ1-42 oligomers. Despite complex structure, and cytotoxic nature of AmB, the ability of AmB to recognize soluble oligomers of Aβ1-42 is significant. This macrolide antibiotic is readily available and functional groups around the skeleton can be selectively modified to yield synthetic analogues with reduced toxicity and increased functional utility.[30–33]

3. Conclusions

We have demonstrated that AmB can bind to two distinct, soluble secondary structure conformations of Aβ1-42, that is, unordered oligomers and ordered oligomers. The calculated binding constants are similar in both cases. However, a threefold increase in the number of binding sites for AmB was observed upon the transition from an unordered to a β-sheet soluble conformation of Aβ1-42. Importantly, AmB had no measurable effect on the kinetics of the amyloid oligomerization process. Thus, monitoring chiroptical responses of both the ligand and the peptide allowed for the differentiation between the binding of the small molecule to Aβ-aggregates and the inhibition of amyloid aggregation.

4. Experimental

4.1. Materials

AmB (80% preparation from *streptomyces*), TRIS and NH_4OH solution (>25%) were purchased from Sigma Chemical Co. (St. Louis, MO), and used as received. Milli-Q-system was used to purify water that was utilized throughout this work. Aβ1-42, NaOH-treated preparation, was from Recombinant Peptide, Inc. (Athens, GA).[34]

4.2. Methods

CD spectra were acquired on Jasco J-815; far-UV spectra (190–250 nm) were recorded using 0.1 cm quartz cells; AmB CD transitions (250–400 nm) were recorded using 1 cm quartz cell. Spectra were recorded at room temperature and 1 nm resolution with a scan rate of 100 nm/min. Usually two scans were acquired and averaged for each sample. Raw data were manipulated by subtraction of appropriate background spectra, followed by smoothing using manufacturer provided software. Data expressed as a mean residue ellipticity, (deg $dmol^{-1}$ cm^2) for Aβ1-42, and either as CD intensity, θ (mdeg), or molecular ellipticity, $\Delta\varepsilon$ (M^{-1} cm^{-1}), for AmB. Absorbance measurements were performed on Agilent UV–visible instrument using 1 cm quartz cells. Static and dynamic light scattering measurements were performed at 23.0 °C on a light scattering apparatus built using the following main components: He–Ne laser (35 mW, 632.8 nm, Coherent Radiation), manual goniometer and thermostat (Photocor Instruments), multitau correlator, and APD detector and software (PD4042, Precision Detectors). All experiments were performed at the scattering angle of 90°.

4.3. AmB stock solution preparation

AmB was dissolved in DMSO at 20 mg/ml, and subsequently diluted into 10 mM TRIS pH 7.4 buffer to obtain 0.04 mg/ml stock

solution, which was used for all titration experiments. AmB stock solution was prepared fresh before each experiment. The handling of AmB, that is, sample preparation and spectroscopic measurements were done in a dim light environment.

4.4. Aβ1-42 peptide solution preparation

Aβ1-42 was stored at −20 °C; prior to experiments it was allowed to equilibrate at room temperature for 45 min–1 h, and treated according to the manufacturer's instructions. Briefly, the peptide was dissolved in 1%NH_4OH at 1 mg/ml concentration, sonicated for 1 min to give Aβ1-42 stock solution. In order to obtain a random coil conformation, the freshly prepared peptide stock was diluted into 10 mM TRIS buffer (pH 7.4) and gently mixed by dispensing it with a pipette tip (vortexing, sonication and centrifugation were avoided) to obtain 25 μM Aβ1-42 solution, pH8.7, which was subsequently titrated with the AmB stock solution or 10 mM TRIS (1% v/v DMSO) as a control. The final concentration of DMSO in all cases was 0.015% v/v. A β-structure soluble conformation of Aβ1-42 was obtained by incubating the above 25 μM Aβ1-42 solution at room temperature for 48–72 h. Subsequently, this solution was titrated with freshly made solution of AmB to estimate the affinity of AmB towards the ordered, β-structure-rich conformation of Aβ1-42 oligomers.

Acknowledgments

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