Thioflavin T Promotes Aβ(1−40) Amyloid Fibrils Formation

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Letter

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Thioflavin T Promotes Aβ(1−40) Amyloid Fibrils Formation

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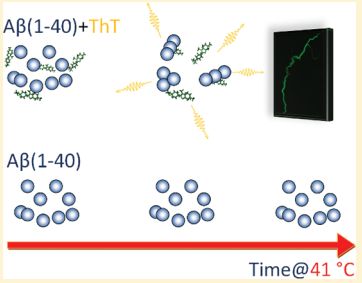
Supporting Information

ABSTRACT: Fibrillogenesis of the small peptide Aβ(1−40) is considered to be the hallmark of Alzheimer's disease. Some evidence indicates small oligomers, rather than mature fibrils, as the key cytotoxic agents. The fluorescent dye Thioflavin T (ThT) is often used to detect amyloid deposits in both in vivo and in vitro experiments, and it is used to study kinetic measurements, under the fundamental hypothesis that this probe does not influence the aggregation processes. We report experimental data showing that ThT may promote the Aβ(1−40) peptide amyloid aggregation changing solvent−peptide interactions and stabilizing more ordered β-like conformation. This finding has a two-fold importance: It is a fundamental warning in all fibrillation experiments where ThT is used as fluorescent probe, and it suggests that ThT, accelerating fibril formation, could be used to reduce the presence of transient small oligomers, thus interfering with the pathogenic impact of Aβ peptide.



SECTION: Biophysical Chemistry and Biomolecules

The study of aggregation processes and, in particular, of amyloid formation is nowadays of fundamental interest because the creation and the deposition of these aggregates are associated with high impact human disorders like Alzheimer's, Parkinson's, Creutzfeldt-Jakob diseases and type 2 diabetes.[1,2] Amyloid fibrils are ordered elongated aggregates stabilized by an highly organized H-bond pattern originating from a protofilament backbone consisting of a single double-pleated β-sheet.[3] In many cases, pathological (as well as non-pathological) amyloid aggregation was found to occur via the formation of multiple kinetic intermediates.[2,4] In such a complex aggregation landscape, it has been suggested that the generic amyloid conformation may constitute a deep and sharp energetic minimum representing the most stable state that multiprotein systems can adopt.[2,5] The detailed comprehension of aggregation kinetics is important for the determination of the diverging point between regular protein folding and protein aggregation processes and to understand what causes contribute to this nonfunctional end.[6] Analysis of aggregation kinetics is often performed using spectroscopic methods in a time-resolved fashion. The formation and the order of the kinetic intermediates, the time scales of the processes, and the dimension of the aggregates are all fundamental questions that can only be clarified with a detailed kinetic analysis of the aggregation processes.[6−8] In particular, the detection of transient species like protofibrils and small oligomers appears to be essential because nowadays they are considered to be the prime toxic agents in amyloid diseases.[4,9−12]

One of the most common methods is the analysis of Thioflavin T (ThT) fluorescence kinetics as a probe for the detection of amyloid fibrils. Indeed, as it is well-assessed, the fluorescence signal of ThT in aqueous solution is almost vanishing for the presence of highly efficient internal non-radiative channels, whereas the fluorescence intensity shows a dramatic increase in the presence of amyloid fibrils both in vivo and in vitro.[8,13−15] The mechanism by which ThT recognizes and binds fibrils, amyloid plaques in diseased tissues or other β-rich self-assembled peptides was matter of debate in the last years.[16−20] The main proposed models involve the presence of ThT micelles,[17] formation of ThT dimers,[18] or highly directional monomer binding into β-sheet structured cavities with ThT long axis parallel to the elongation axis of the fibrils.[16] However, this last model was recently confirmed by experimental and theoretical works, and it is the most accepted one.[21−24]

There is also a renovated attention for this dye because it was found to slow down aging processes in the model organisms *C. Elegans*.[25] These unexpected health benefits seem to be related to ThT capability of suppressing protein-aggregation-associated paralysis in toxic protein models in multiple tissues.[25] It was intriguingly suggested that the presence of ThT may affect the amount or the size of proteins prone to aggregate.[26]

Notwithstanding the large use of ThT to analyze "in situ" aggregation kinetics, the fact that ThT presence does not alter the amyloid formation pathway appears to be rather assumed than demonstrated.[15]

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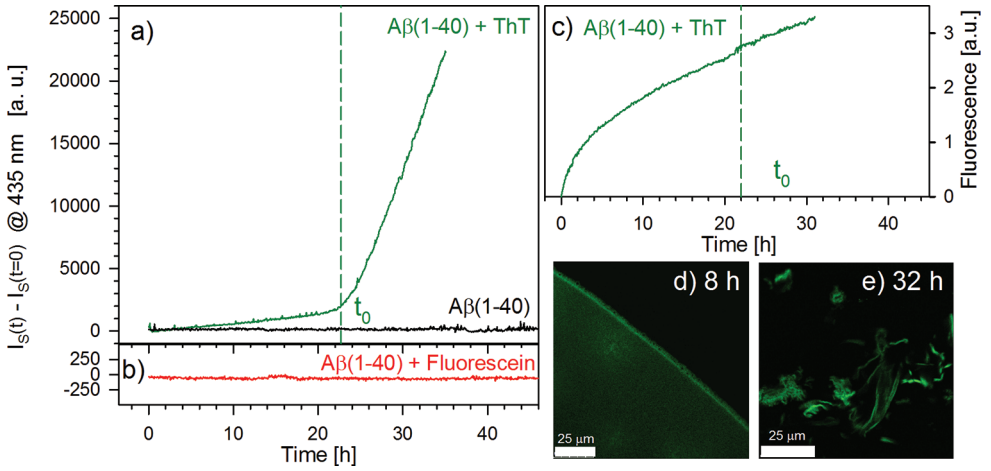

Figure 1. (a,b) Rayleigh scattering intensities at 435 nm as a function of time for samples incubated at 41 °C: Black line refers to the sample of Aβ(1−40) at a concentration of 150 μM; green line corresponds to a similar sample with the addition of 20 μM of ThT; red line corresponds to a similar sample with the addition of Fluorescein. (c) Fluorescence intensity at 490 nm as a function of time in the Aβ(1−40) sample containing 20 μM of ThT. The vertical dashed green lines represent the time of the abrupt increasing of the scattering signal (t_0). (d,e) Two-photon fluorescence microscopy images of the Aβ(1−40) sample with ThT after 8 and 32 h of incubation, respectively. The white bars represent 25 μm.

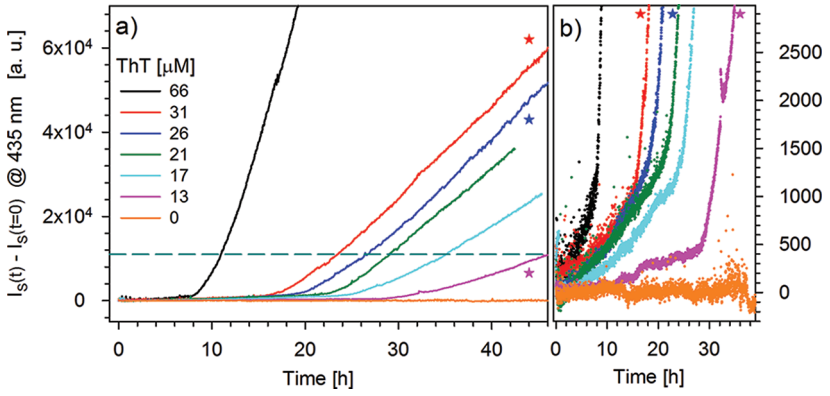

Figure 2. (a) Rayleigh scattering intensities at 435 nm as a function of incubation time for seven representative samples with different ThT concentrations. (b) Enlarged scale of panel a. For both panels, the curves labeled with stars refer to samples containing recrystallized ThT.

In the present Letter, we show the effect of ThT in solution on the fibril formation process of Amyloid β(1−40), [Aβ(1−40)], a short peptide universally known for its implication in the age-related Alzheimer's disease. Its self-assembly into amyloid fibrils is nowadays considered to be the hallmark of this pathology.[1,9,27] We measured Aβ(1−40) aggregation kinetics in the presence/absence of ThT. We wish to point out that all observed kinetics are not influenced by any mechanical stress (agitation or stirring) and occur under conditions in which interfacial effects can be neglected. Our results clearly show that under the observed conditions (T = 41 °C and pH 7.4) the Aβ peptide alone does not give rise to supramolecular association, but, in disagreement with common thought, the presence of ThT in solution induces Aβ aggregation. It is possible to argue that the presence of ThT induces some more ordered β-like conformation in the peptide, which alters the protein−solvent interactions so that the system evolves toward the observed association.

Figure 1a shows the time evolution of Rayleigh scattering intensity measured at 435 nm for 150 μM of Aβ(1−40) in solution at pH 7.4, incubated at 41 °C for 46 h (black line). The green line refers to the measurements on Aβ(1−40) at the same concentration in the same buffer containing ThT (20 μM) (hereafter Aβ-ThT). As it is observed, for the Aβ(1−40) sample the scattering intensity remains constant during the incubation time. This behavior indicates that the average size of the particles in solution remains unchanged at least up to 46 h. Interestingly, in the presence of ThT (green line), the scattering signal shows a two-step kinetics: In the first phase, it slowly increases almost linearly, then it undergoes an abrupt growth after about 23 h (t_0). This behavior resembles the biphasic kinetics consisting of a lag and a growth phases, which usually indicates nucleated polymerization mechanisms in Aβ fibrillation.[28] In panel b, we also report, as a control, the corresponding scattering measurement on an Aβ sample containing Fluorescein at a concentration of 25 μM, in the place of ThT. As can be seen, as well in the Aβ(1−40) sample, changes in the scattering signal are not observed indicating that the increase in scattering in the presence of ThT is not barely due to the presence of small organic molecules in solution. In Figure 1c, the time evolution of ThT fluorescence intensity in the Aβ-ThT sample, simultaneously measured with the scattering signal showed in panel a, is reported. In parallel with the first slight increase in scattering intensity ($t < t_0$) ThT fluorescence increases as a function of time and continues its growth also after t_0, where a linear dependence is recorded. In panel d, a two-photon fluorescence microscopy image representative of the Aβ-ThT samples in the lag phase is

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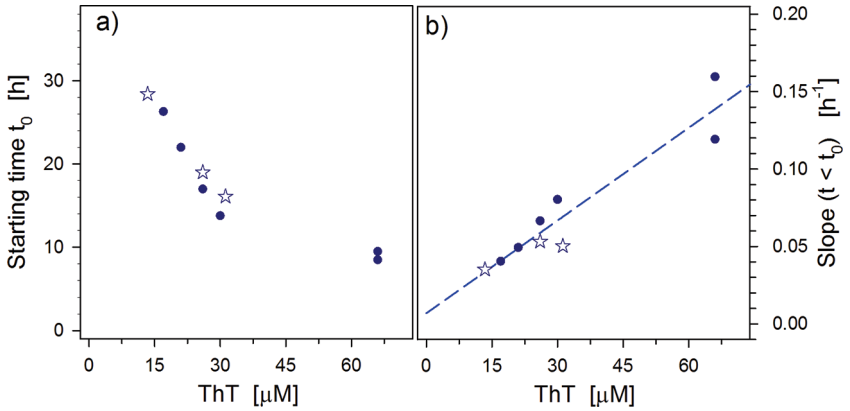

Figure 3. (a) Starting time t_0 as a function of ThT concentration. (b) Slope of the first part ($t < t_0$) of scattering kinetics as a function of ThT concentration. The dashed line represents the best linear fit to the points. For both panels, stars refer to samples containing recrystallized ThT.

shown: It is possible to observe the edge of a drop of Aβ-ThT solution incubated for 8 h. ThT fluorescence signal (green pixels) is uniformly distributed in the sample,[29] suggesting the presence of small aggregate structures that bind ThT and whose size is below the instrumental resolution (~200 nm). In line with the small scattering increase reported for this sample in panel a, also Forster recovery after photobleaching (FRAP) measurements (data not reported) confirm the presence of oligomers (diffusion coefficient D in the range of 10−20 μm^2 corresponding to particles with an average hydrodynamic radius of 26 ± 9 nm). In panel e, a representative image of the Aβ-ThT sample incubated for ~32 h is reported. In this stage, the presence of large fibrils is clearly evident.

These results indicate that under the observed experimental conditions the presence of ThT in solution triggers (or at least expedites) Aβ(1−40) aggregation. This process occurs via the growth of small aggregates in the lag phase, which could be involved in nucleation mechanisms, leading to large fibrils formation during the rapid growth phase observed using Rayleigh scattering for $t > t_0$.

In Figure 2, the time evolution of ThT fluorescence intensity in Aβ(1−40) samples at different ThT concentrations ([ThT]) in the range 0−66 μM is reported. To guarantee that the observed effect of ThT was not due to the presence of impurities in the commercial ThT (purity of 65−75%), we performed measurements (labeled with stars) also with ThT purified by a recrystallization procedure where the organic proton-containing impurities and excess salts were removed. (See the Supporting Information.) All measured scattering kinetics present the same biphasic trend (except for data with [ThT] = 0): a almost linearly increase in the first hours and a subsequent abrupt growth after a time t_0, which depends on ThT concentration. Interestingly, as it is pointed out in Figure 3a, t_0 decreases as [ThT] increases. Moreover, the slope of the first increasing part of scattering data ($t < t_0$) increases linearly with [ThT]. (See Figure 3b.)

Figure 4 shows the behavior of ThT fluorescence as a function of incubation time, normalized at 3 h. Data are shown until the corresponding scattering intensities are below the dashed horizontal line of Figure 2a to ensure that high scattering signals do not influence the slope of fluorescence. In all samples the ThT fluorescence intensities grow almost monotonically as a function of time. Moreover, we note that in the first part the data perfectly overlap and after about 5 h the signal increase depends on [ThT].

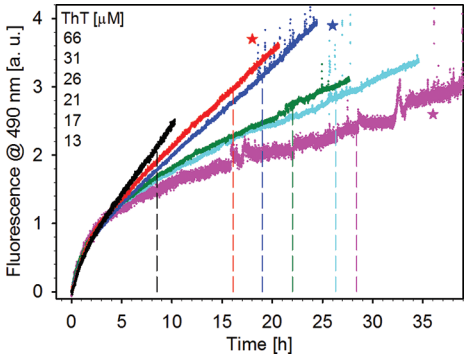

Figure 4. ThT fluorescence intensities at 490 nm normalized at 3 h as a function of incubation time for six representative samples with different ThT concentrations. The vertical dashed lines correspond to the times t_0 when corresponding signals of Figure 2 show a dramatic scattering increase. Stars refer to samples containing recrystallized ThT.

Our results clearly indicate that ThT induces Aβ(1−40) peptide aggregation leading to the formation of large fibrillar structures observed using two-photon microscopy (Figure 1e). Indeed, in the presence of ThT, the average dimensions of objects in solution begin to increase upon incubation, whereas in the absence of ThT no variations are observed. (See Figure 1.) The rate of aggregate formation and the lag time, t_0, critically depend on ThT concentration (see Figures 2 and 3), and the progressive increase in ThT fluorescence (Figure 4) indicates that structures that bind this dye are progressively formed. The fact that ThT addition to the Aβ samples favors its aggregation is confirmed by the experimental observation that the starting time t_0 approaches infinity as [ThT] approaches to zero. (See Figure 3a.) That is, the more the ThT concentration is increased the more the Aβ aggregation speeds up. ThT is known to bind fibrillar structures, and it was suggested that under certain conditions its fluorescence growth may be related to the formation of β-aggregate structures, and in these instances a small fluorescence intensity increase, related to a protein secondary structure change, is observed.[14−16,30−32] Therefore, the increase in the ThT fluorescence signal suggests the formation of β-aggregates. (See Figure 4.) The time dependence of fluorescence intensity growth is the same for all samples regardless of the amount of ThT. In particular, the ThT fluorescence intensity reported in Figure 4 overlaps in the first 5 h, showing the same rate for all samples. After that, the

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kinetic behavior measured by fluorescence depends on the ThT concentrations. Possible peptide conformational changes toward structures able to bind ThT molecules (present in solution at lower concentration than Aβ(1−40), from 1:12 to 1:2 molar ratio) could explain the common behavior of fluorescence signal.

The effect of promoting Aβ(1−40) aggregation has to be a feature of ThT molecules; indeed the addition of Fluorescein in the solution (an aromatic molecule with similar molecular weight as ThT) does not trigger any effects in the first 46 h. (See Figure 1b.) Interactions between ThT and the peptide seem somehow specific, and the effect could be related to the symmetry of the molecule and specific side groups. Hydrophobic and electrostatic interactions between Aβ and ThT may favor supramolecular association. Also, ThT charge and conformation may favor protein−dye interaction.[33] In particular, aggregates formation may be due to the interaction between ThT aromatic rings and nonpolar aromatic side chains of Aβ molecules in solution.[15] Moreover, Aβ aggregation may also be affected by the possible presence of ThT micelles with hydrophobic interiors and positively charged N pointing toward the solvent.[20]

It should be noted that the same effect was not found in amyloid fibril formation from globular proteins.[30−32,34] This may be explained taking into account that the free energy profile of a natively unstructured protein is quite different than for globular proteins.[35] The Aβ(1−40) chain has a specific distributions of polar and nonpolar residues, and it is known to have a disordered structure in aqueous solution[27,36] so that various conformations are available at the equilibrium for a single molecule, giving to reactive groups in the chain the possibility of being exposed and to interact with correspondent groups of the ThT dye. In particular, natively unfolded proteins are expected to populate conformations that are different from each other on all possible length scales.[35] In this situation, it is possible to infer the existence of a thermodynamic drive toward the stabilization of these groups by interaction with ThT specific groups that act as a template.[4] The presence of ThT molecules in solution may induce conformational changes trapping a specific, more ordered, conformation prone to supramolecular assembly. Sterical and chemical properties of the ThT may modulate the peptide conformation, with similar mechanisms to the ones that usually drive the binding of this dye to already formed amyloid structures. The dye may mediate a selection process toward a specific structure whose presence favors the self-assembly process so that this effect could be considered as a possible example of template-dependent fibrillation.[4] This was shown for other small molecules able to stabilize different intermediates during the supramolecular assembly modulating the fibrillation pathway and determining the final morphology of the aggregates.[4,11,37−41] In this report, we demonstrated that under the experimental condition used the Aβ(1−40) peptide does not aggregate except when ThT is present in the solution. Upon ThT addition, the small peptide showed a typical biphasic kinetic (lag and growth phases) toward the formation of fibrils able to stain ThT. We wish to note that the kinetics profiles and the lag times, t_0, are highly repeatable. This does not exclude the presence of a stochastic nucleation mechanism, which is usually featured by the absence of data repeatability. Stochastic mechanisms cannot be easily discriminated for their intrinsic complexity. For example, it was shown that concentration-dependent variation of Aβ fibrillation reactions becomes progressively homogeneous as the concentration is raised.[28] Here it is not possible to infer a simple two-step aggregation mechanism in which small β-aggregates formed in the first phase act as nuclei for a subsequent homogeneous process. Whereas ThT emission is related to conformational properties of the aggregates, light scattering intensity takes into account the average size of all aggregates in solution.[31] The combination of the two methods can be used to outline different features of the amyloid growth, and in this specific case it indicates more complex underlying processes: The Rayleigh scattering measurements (Figure 2) and the fluorescence data (Figure 4) do not show any direct correlation. Further studies are needed to detect the intermediate steps and aggregated species involved in the observed supramolecular assembly.

This important result indicates that ThT can act as template to change the Aβ(1−40) random conformation in a β-aggregated form prompt to form amyloid fibrils. This could be important in the viewpoint that ThT may favor the conversion of possible toxic Aβ oligomers into less reactive amyloid fibrils. This result is a fundamental warning for experiments where ThT is used as a normal amyloid fluorescent probe with the assumed hypothesis of no interaction with the peptide.

EXPERIMENTAL METHODS

Aβ(1−40) was purchased from Biopeptide. Before dissolution, the peptide was disaggregated according to a slightly modified Fezoui protocol.[42] (See the Supporting Information.) Samples were prepared in cold room dissolving Aβ(1−40) in 50 mM phosphate buffer pH of 7.4 containing ThT. The solution was filtered into a cuvette using 0.20 μm filter (Sartorius no. 17761). The first measurement is captured ∼5 min after the Aβ dissolution. ThT (T3516) and Fluorescein (32615) were purchased from Sigma Aldrich and used without further purification. When needed, purified ThT was obtained by using a recrystallization protocol. (See the Supporting Information.) For both types of ThT, the same molar extinction coefficient of 36 000 M^{-1} cm^{-1} was used to obtain the ThT concentrations.[14] Fluorescence measurements were performed in standard right-angle geometry on a 0.5 × 1.0 cm PMMA semimicro UV-cuvette (BRAND) under excitation at 435 nm. Rayleigh scattering signal at 90° was simultaneously measured as the maximum of the elastic peaks of excitation light. (More details of experimental setup are reported in the Supporting Information.) During the measurements, the Aβ(1−40) solution was not influenced by any mechanical stress (agitation or stirring). Measurements were repeated several times to ensure the reproducibility of experiments. We note that for measurements up to 50 h the ThT fluorescence at pH 7.4, 41 °C is not affected by hydroxylation process, which could invalidate the analysis of fluorescence kinetic.[43]

ASSOCIATED CONTENT

Supporting Information

Description of recrystallization of ThT, the protocol used to disaggregate the Aβ peptide, and more details of experimental setup and methods are reported. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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