Pentameric Thiophene as a Probe to Monitor EGCG’s Remodeling Activity of MatureAmyloid Fibrils: Overcoming Signal Artifacts of Thioflavin T

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ABSTRACT: Thioflavin T fluorescence is a gold standard probe for the detection of amyloid fibrils. Herein, we showed that mature amyloid fibrils incubated with polyphenol epigallocatechin gallate (EGCG) present a fast reduction of the thioflavin T fluorescence, which is not related to remodeling activity. We propose the use of the pentameric thiophene fluorescence for monitoring the polyphenol remodeling activity.

INTRODUCTION

Accumulation of amyloid fibrils is the hallmark of amyloid diseases responsible for about one in a thousand deaths in developed countries. Among these often-fatal diseases are Alzheimer’s and Parkinson’s diseases, type 2 diabetes, and several other amyloidosis. Amyloid fibrils share a typical conformation rich in cross-β structures formed by intertwined layers of β-sheets extending parallel to the fibril axis. Part of the main structural features of the amyloid fold extracted from in vivo patients can be recapitulated by in vitro aggregates of purified recombinant amyloidogenic proteins. This phenomenon helps the study of molecular mechanisms related to amyloid aggregation. Moreover, the study of amyloid aggregation in vitro allowed the discovery, by high throughput screening, of small molecules to block the amyloid aggregation or disassemble the preformed mature amyloid fibrils. Several antiamyloidogenic small molecules are currently under investigation and clinical trials, among them is epigallocatechin gallate (EGCG) (Figure S1A), the main catechin of green tea. EGCG interacts directly with several amyloidogenic proteins in vitro, blocking the fibrillization process and driving the remodeling of mature amyloid fibrils. The activity of EGCG against amyloidogenic proteins was extensively investigated by the use of several biochemical and biophysical techniques such as nuclear magnetic resonance, Raman spectroscopy, transmission electron microscopy, atomic force microscopy, among others. However, the most used approach to detect amyloid formation and disassembly in vitro is the fluorescence of thioflavin T (ThT) (Figure S1B). ThT is a benzothiazole salt widely used to visualize and quantify the amyloid fold both in vivo and in vitro. The molecular mechanism to explain the increase in the fluorescence emission of ThT upon amyloid binding is controversial, but several studies point to the restriction of the free rotation of ThT rings. Even after being a gold standard probe to quantify amyloid in vitro, ThT fluorescence presents some critical limitations such as specificity, interference with aggregation kinetics, and the dependence on the ionic strength of the solution utilized. Other groups and ourselves have presented evidence that EGCG competes with ThT for some amyloid binding sites. Luminescent-conjugated polythiophenes have been used to study protein aggregates in vitro, in situ, and in vivo. Among the conjugated polythiophenes, pentameric thiophene (pentameric formyl thiophene acetic acid (PFTAA), Figure S1C) exhibits conformation-dependent spectral properties, and when immobilized into agarose beads, it was able to pull down amyloid aggregates. Herein, we compare the ThT and PFTAA fluorescence as a readout of the EGCG remodeling activity against mature amyloid fibrils.

RESULTS AND DISCUSSION

The protein used as a model herein was recombinant mature amyloid fibrils of alpha-synuclein (α-synuclein), the main protein related to Parkinson’s disease. The mature fibrils of α-synuclein were incubated with 0.25 mM epigallocatechin gallate (EGCG) and 0.125 mM pentameric thiophene (PFTAA) for 0 and 24 hours. The fluorescence of ThT and PFTAA were measured before and after incubation with EGCG and PFTAA. The results showed a fast reduction of the thioflavin T fluorescence, which is not related to remodeling activity. We propose the use of the pentameric thiophene fluorescence for monitoring the polyphenol remodeling activity.

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Figure 1. Effect of EGCG on the ThT and PFTAA fluorescence of α-synuclein amyloid fibrils. (A) Experimental scheme of the centrifugation/wash protocol. (B) ThT fluorescence or (C) PFTAA fluorescence of the samples incubated for 0 or 24 h in the absence or presence of EGCG. The use of the centrifugation/wash protocol is denoted by (+). The buffer used for all assays was 50 mM phosphate buffer pH 7.4, 150 mM NaCl (25 °C). [α-synuclein] = 5 μM and [EGCG] = 30 μM. For the ThT fluorescence, the ThT concentration used was 20 μM, Ex: 450 nm, and Em: 477 nm. For the PFTAA fluorescence, the PFTAA concentration used was 20 μM, Ex: 450 nm, and Em: 550 nm. Transmission electron microscopy of α-synuclein amyloid fibrils incubated in the absence (D) or presence of EGCG for 0 (E) or 24 h (F). Bar = 500 nm.

Figure 2. Titration of EGCG at α-synuclein amyloid fibrils monitored by ThT or PFTAA. Mature α-synuclein amyloid fibrils (10 μM) were incubated with 20 μM ThT (A) or PFTAA (B), and the fluorescence was measured (green spectra). A titration of EGCG ranging from 5 to 25 μM was performed, and the fluorescence of ThT or PFTAA was measured immediately. As a negative control, no fibrils were added to the ThT or PFTAA solution (black spectra). Panel C presents the area of each spectrum at increasing EGCG concentrations.
α-synuclein were characterized by transmission electron microscopy and circular dichroism (Figure S2A,B, respectively). We observed an immediate decrease in ThT (20 μM) fluorescence when α-synuclein fibrils (5 μM) were incubated with EGCG (30 μM) (Figure 1B, time 0 h, wash −). As reported before,10 we took advantage of a methodology that allows us to quantify the ThT fluorescence after performing a series of fibril centrifugations and washes to remove any soluble EGCG before each ThT measurement (Figure 1A). We observed that the ThT fluorescence of fibrils incubated with EGCG followed by the centrifugation/wash protocol returns to the same level as that of untreated fibrils (Figure 1B, time 0 h, wash +).

On one side, after 24 h incubation, even after the centrifugation/wash protocol, the ThT fluorescence of EGCG treated fibrils remains low (Figure 1B, time 24 h, wash +). We confirmed by transmission electron microscopy that fast incubation (time 0 h) with EGCG causes no morphological changes into the amyloid fold (compare Figure 1D with 1E). In contrast, after 24 h incubation, an intense amyloid fibril disaggregation remodeling activity was observed (Figure 1F). These data suggest that the ThT fluorescence of amyloid fibrils at zero time point does not reflect the reality when EGCG is added into the solution. A different scenario was observed when the ThT probe was replaced by PFTAA (Figure 1C). The PFTAA fluorescence was not affected by EGCG incubation at zero time point (Figure 1C, time 0 h, wash −). Next, we incubated α-synuclein fibrils (10 μM) with ThT or PFTAA (20 μM) and performed the titration of EGCG (Figure 2). We observed a dose–response decrease in ThT fluorescence immediately after incubation with EGCG (Figure 2A). On the other side, the presence of EGCG does not interfere with the fluorescence of PFTAA (Figure 2B,C). As expected, this fast incubation, which was about 5 min considering the sample manipulation, is insufficient to cause morphological changes in α-synuclein fibrils (compare Figure 2D with 2E). It is important to note that the increase in the fluorescence of ThT after incubation with amyloid fibrils is much higher when compared with PFTAA (1000-fold vs 4-fold, compare the black vs green spectra of Figure 2A,B). However, as pointed herein, a probe with high sensitivity does not mean that it is superior to others. We performed EGCG titration in a similar way as described in Figure 2, keeping the same concentration of α-synuclein fibrils but this time changing the concentration of ThT (Figure 3A) or PFTAA (Figure 3B). Again, all experiments were performed at zero time point incubation. Surprisingly, when low concentrations of ThT were used, the reduction of the ThT fluorescence was prevented upon EGCG incubation (Figure 3A, compare circles with inverted triangles). A possible explanation for this observation may be related to the existence of more than one ThT binding site with different affinities in α-synuclein fibrils. At low ThT concentrations, the first binding site with higher affinity is occupied, and EGCG cannot bind to this site. Only with higher ThT concentrations, the second binding site is occupied, being susceptible to EGCG competition. Another hypothesis is that EGCG disrupts the micelles of ThT formed only at high concentrations of ThT.29 It was showed by Roy and colleagues through NMR that EGCG binds to the same site as ThT into amyloid fibrils.30 Probably this feature responds to the immediate reduction of ThT upon EGCG incubation.

Finally, we performed a kinetics experiment measuring the ThT or PFTAA (20 μM) fluorescence incubating mature α-synuclein fibrils (5 μM) in the absence or presence of EGCG (30 μM) for 4 h (Figure 4). For both probes, the fibrils incubated in the absence of EGCG showed a slight reduction in the fluorescence (30 %) that is usually observed in plate reader experiments due to adsorption to the plastic surface multwell plates (Figure 4, open symbols).30 The incubation with EGCG caused a fast reduction in the ThT fluorescence (Figure 4, hatched circles). The PFTAA probe allowed us to observe that the remodeling activity of EGCG takes place

Figure 3. Titration of amyloidogenic probes at α-synuclein amyloid fibrils monitored incubated with EGCG. Mature α-synuclein amyloid fibrils (10 μM) incubated with different concentrations of ThT (A) or PFTAA (B) were incubated with increasing concentrations of EGCG, and their fluorescence was measured at zero time point.

Figure 4. Kinetics of α-synuclein amyloid fibril remodeling monitored by ThT or PFTAA fluorescence incubated with EGCG. α-synuclein amyloid was incubated in the absence (open symbols) or presence of EGCG (hatched symbols) at 37 °C, and the ThT (circles) or PFTAA (squares) fluorescence was monitored according to the time. [α-synuclein] = 5 μM and [EGCG] = 30 μM. For the ThT fluorescence, the ThT concentration used was 20 μM, Ex: 450 nm, and Em: 477 nm. For the PFTAA fluorescence, the PFTAA concentration used was 20 μM, Ex: 450 nm, and Em: 550 nm.
approximately after 1 h incubation and occurs during the following hours of the experiment (Figure 4, hatched squares).

As pointed in Figure 2, PFTAA showed a low increase in the fluorescence quantum yield upon amyloid incubation, indicating that this probe has modest sensitivity. Moreover, it was previously demonstrated that PFTAA changes the aggregation kinetics of the Aβ42 peptide. The second most used probe for amyloid detection after ThT is Congo red. However, Congo red has even more limitations than PFTAA. Usually, aggregation kinetics experiments must be performed in the absence of Congo red since this probe blocks the amyloidogenic aggregation of most proteins. Moreover, it was demonstrated that amyloid incubated with EGCG for 24 h induced a red shift absorbance of Congo red that was almost identical to fibrils without EGCG.

Why do ThT and PFTAA have different amyloid fibril binding modes? To definitively answer this important question, detailed atomic models of ThT, PFTAA, and EGCG bound to mature fibrils are necessary. However, some clues can be gathered from biding experiments. It was shown through structural simulations that the ThT binding into Aβ peptide aggregates occurred in two different binding sites. It was subsequently suggested that a surface binding mode and a channel insertion binding mode of ThT to amyloid fibrils can result in different spectroscopic properties. Most ThT binding occurs by surface contact stabilized by ionic interactions, allowing fast relaxation, and consequently, a low fluorescence quantum yield. Fewer ThT molecules interact with amyloid fibrils through a channel insertion mode, where hydrophobic interactions are the dominant forces. The channel insertion binding mode represents the strongest emissive population of ThT. In other words, the hydrophobic interactions play a major role in the observed increase in the ThT fluorescence upon amyloid binding. In contrast, ionic interactions between the anionic carboxyl groups of PFTAA and the cationic ε-amino groups of the side chain of amino acids seem to be determinant PFTAA binding. Bieschke and colleagues showed that the gallocate moiety of EGCG (D ring, Figure S2A) is critical for the efficient amyloid remodeling activity. We previously showed that EGCG oxidation drives amyloid remodeling by hydrophobic interactions. Together, these pieces of evidence suggest that the hydrophobic interactions are the main determinants for EGCG amyloid binding and remodeling. We hypothesize that EGCG and ThT compete for the hydrophobic pockets present in the amyloid structure. On the other side, EGCG poses no competition to PFTAA since this probe binds to amyloid fibrils mainly by ionic interactions.

We conclude that the fast initial decrease of the ThT fluorescence from mature amyloid fibrils incubated with EGCG is an artifact and cannot be interpreted as remodeling activity. We speculate that EGCG competes with ThT for channel pockets of mature fibrils. We anticipate that this artifact is not restricted to polyphenol EGCG since we observed this feature with diverse other small molecules (data not shown). To address this issue, we suggest some alternatives: (i) the use of the centrifugation/wash protocol since most amyloid fibrils are easily pelleted by centrifugation; (ii) the use of the PFTAA probe; (iii) the use of ThT at low concentrations; and (iv) the use of other techniques, for example, transmission electron microscopy, to follow the remodeling activity of polyphenolic compounds or any other small molecule.

### METHODS

**EGCG.** The EGCG (Sigma-Aldrich) degree of purity was evaluated through a reversed-phase chromatography system with a binary gradient of elution and showed >95 % purity. Its identity was confirmed by electrospray-ion trap mass spectrometry (Bruker Amazon SL model) as described previously.

**Thioflavin T (ThT).** ThT was purchased from Sigma and diluted in ultrapure water to a final concentration of 2 mM.

**Pentameric Thiophene (PFTAA) Synthesis.** PFTAA was synthesized as previously described.

**Preparation of α-Synuclein.** α-Synuclein was purified as described previously. Briefly, lyophilized α-synuclein was resuspended in phosphate-buffered saline (PBS), filtered through a 0.22 μm filter, and centrifuged using a Centricon with a 100 kDa cutoff. Then, the homogeneity of the α-synuclein monomers was analyzed by size-exclusion chromatography (Superdex 200 10/300 GE Healthcare), and the identity of α-synuclein was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Autoflex Speed model). For size-exclusion evaluation, 10 μL of each sample was injected at a 0.05 mL/min flow rate, and elution was monitored by fluorescence (λex = 280 nm; λem = 320 nm). To obtain the mature amyloid fibrils, monomeric α-synuclein was incubated (140 μM) in PBS pH 7.4 with 0.02 % NaN3 with agitation at 37 °C for 6 days. The fibrils were centrifuged (16 000 g for 10 min at 4 °C), the supernatant was removed, and the pellet was stored at −20 °C until use.

**Centrifugation/Wash Protocol to Remove Unbound EGCG.** Aliquots (200 μL) of α-synuclein fibril (5 μM) samples in the absence or presence of EGCG (30 μM) were centrifuged (16 000 g for 10 min at 4 °C) to obtain a pellet. The pellet was washed with 400 μL of 50 mM phosphate buffer (pH 7.4) and 150 mM NaCl, and the solution was centrifuged again (16 000 g for 10 min at 4 °C). The pellet was resuspended in 50 mM phosphate buffer (pH 7.4) and 150 mM NaCl with 20 μM thioflavin T or 20 μM of pentameric thiophene.

**Circular Dichroism.** The measurements were performed with a Jasco J-715 (Jasco Corp., Tokyo, Japan) spectropolarimeter using a 0.01 mm path length quartz cuvette. CD spectra were acquired using the following parameters: wavelength 260–290 nm, bandwidth 1 nm, speed 100 nm min⁻¹, and response time 0.4 s. Spectra were recorded with an average of 3 scans, and the baseline (buffer alone) was subtracted from the spectra. The sample was analyzed in PBS (pH 7.4) with 0.02 % NaN3 at 25 °C.

**Transmission Electron Microscopy.** The samples were prepared as described by Azevedo and colleagues.

**Fluorescence Assays.** The fluorescence assays described in Figures 1–3 were performed in an ISS spectrophotometer with a 1 nm slit width for excitation and emission. The amyloid fibrils (5 or 10 μM) were diluted in PBS (pH 7.4). The fluorescence increase was monitored at 25 °C, with λex = 450 nm and a scan of the emission spectrum from 470 to 570 nm for ThT and with λex = 450 nm and a scan of the emission spectrum from 470 to 680 nm for PFTAA. All experiments were performed at 25 °C. The kinetic experiments (Figure 4) were performed in 96-well plates (Costar # 3631) at 37 °C with shaking (every 5 min for 30 s) containing 20 μM ThT or 20 μM PFTAA. The fluorescence intensity was monitored.
using a SpectraMax Paradigm multimode microplate reader. For ThT binding, the parameters were $\lambda_{ex} = 450$ nm and $\lambda_{em} = 477$ nm, while for PFTAA, they were $\lambda_{ex} = 450$ nm and $\lambda_{em} = 550$ nm. The experiments were repeated at least two times with reproducible results and the bars represent the standard deviation. Figure 4 shows the average of two independent experiments each containing three biological points.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00680.

Chemical structure of EGCG, thioflavin T and pentamer thiophene; characterization of amyloid fibrils of $\alpha$-synuclein (PDF)

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M.K., R.S., L.F., and F.L.P. designed, performed, and analyzed the experiments. F.L.P. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Notes
The authors declare no competing financial interest.

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