

Distinguishing the Effect on the Rate and Yield of A β 42 Aggregation by Green Tea Polyphenol EGCG

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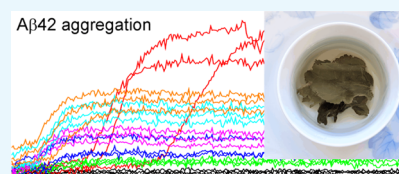


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ABSTRACT: Deposition of A β 42 aggregates in the form of amyloid plaques is a pathological hallmark of Alzheimer's disease. A desired avenue of intervention is the inhibition of A β 42 aggregation. Epigallocatechin gallate (EGCG), the main polyphenol in green tea, has been generally considered an inhibitor of A β aggregation. However, previous experiments focused on the reduction of the amount of A β 42 aggregates, while the effect of EGCG on the rate of A β 42 aggregation was not critically analyzed. Here we performed an experimental evaluation of A β 42 aggregation kinetics in the absence and presence of EGCG at a wide range of concentrations. We found that EGCG reduced thioflavin T fluorescence in an EGCG concentration-dependent manner, suggesting that EGCG reduced the amount of A β 42 fibrils. The effect of EGCG on the rate of A β 42 aggregation appears to be bimodal. We found that higher EGCG-to-A β 42 ratios promoted the rate of A β 42 aggregation, while lower EGCG-to-A β 42 ratios inhibited the aggregation rate. To confirm that the reduction of thioflavin T fluorescence is due to the lowered aggregate quantity, but not due to perturbation of thioflavin T binding to A β 42 fibrils, we probed the effect of EGCG on A β 42 aggregation using site-directed spin labeling. Electron paramagnetic resonance of spin-labeled A β 42 aggregates suggests that high EGCG-to-A β 42 ratios led to a greatly reduced amount of A β 42 fibrils, and these aggregates adopt similar structures as the fibrils in the no-EGCG sample. Potential implications of this work in designing prevention or therapeutic strategies using EGCG are discussed.



INTRODUCTION

Alzheimer's disease, the most common cause of dementia, has two pathological hallmarks: amyloid plaques and neurofibrillary tangles.^{1–3} Amyloid plaques consist of the fibrillar aggregates of A β protein, while tau aggregates make up the neurofibrillary tangles. A β proteins are produced from the proteolytic cleavage of amyloid precursor protein by β - and γ -secretases^{4,5} and are composed of two main isoforms: the 40-residue A β 40 and the 42-residue A β 42. A β 42 is identical to A β 40 with the exception of two extra residues at the C-terminal end. Other variants of A β 42 with both N- and C-terminal heterogeneities also exist.⁶ A β 40 is the most abundant isoform of A β in the brain, several folds more than A β 42, but A β 42 is the major A β isoform in the plaques.^{7–10} Formation of A β fibrils is a result of A β aggregation, a process that has been extensively studied.^{11–17} With amyloid being the central player of the amyloid cascade hypothesis, inhibiting A β aggregation is an obvious avenue for therapeutic intervention. Polyphenols, a group of natural compounds commonly found in fruit and vegetables,¹⁸ have attracted a lot of attention for their antiaggregation activities.¹⁹ Epigallocatechin gallate (EGCG), the main polyphenol compound in green tea, is the focus of this study.

EGCG has been associated with reduced risk for dementia or cognitive impairment. A meta-analysis of 17 studies involving 48 435 participants showed that green tea consumption was associated with a significant reduction in the risk of cognitive disorders.²⁰ Dose–response meta-analysis showed that an increment of 100, 300, and 500 mL/day of tea

consumption was associated with a 6, 19, and 29% lower risk of cognitive disorders.²⁰ Human intervention studies also showed benefits of EGCG. In a randomized, double-blind, placebo-controlled study,²¹ the effect of green tea extract (720 mg, twice daily) was studied in 91 mild cognitive impairment (MCI) subjects for 16 weeks. This study showed that green tea extract improved memory and selective attention in subjects with Mini Mental State Examination-Korea scores of 21–23. Another double-blind randomized study in Japan using 2000 mg/day of green tea powder (containing 220 mg of catechins) for 12 months showed that green tea did not significantly affect cognitive function but prevented the increase of oxidative stress in the elderly population.²²

EGCG has been shown to reduce the amount of amyloid plaques in transgenic animals, but the effect was largely attributed to indirect interactions. Tan and co-workers showed that EGCG reduced plaque load in mice and increased the α -secretase processing of amyloid precursor protein.^{23–25} Levites et al.²⁶ reported that EGCG increased α -secretase processing of amyloid precursor protein via a protein kinase C pathway. *In vitro* studies suggest that EGCG has a direct effect on A β

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aggregation. Previous studies on EGCG and $A\beta$ aggregation have mostly focused on the yield of $A\beta$ aggregation. Bastianetto et al.,²⁷ Sinha et al.,²⁸ and Liu et al.²⁹ showed that EGCG reduced thioflavin T fluorescence of $A\beta$ 42 aggregates in a concentration-dependent manner. Ehrnhoefer et al.³⁰ studied $A\beta$ 42 aggregation in the presence of 10 and 100% EGCGs and showed the reduction of $A\beta$ 42 aggregates using both thioflavin T fluorescence and transmission electron microscopy (TEM). Sironi et al.³¹ showed that EGCG reduced the amount of $A\beta$ 42 fibrils using TEM. There are also studies on the effect of EGCG on preformed $A\beta$ 42 fibrils, and the general conclusion from these studies was that EGCG remodels $A\beta$ 42³² and $A\beta$ 40³³ fibrils to form nonfibrillar aggregates. In two studies,^{28,30} kinetics experiments were performed for $A\beta$ 42 aggregation, but only limited data points were collected and no analysis on the rate of aggregation was performed. Therefore, even though EGCG has been shown to reduce the yield of $A\beta$ 42 aggregation, its effect on the aggregation rate has not been critically evaluated.

In this work, we set out to distinguish the effect of EGCG on the rate and yield of $A\beta$ 42 aggregation using kinetics experiments. In a kinetics experiment, there are two aspects of inhibiting effects: the rate and yield of aggregation. The aggregation rate refers to how fast the $A\beta$ fibrils are formed, and the aggregation yield refers to the amount of $A\beta$ 42 fibrils upon completion of aggregation. $A\beta$ aggregation can be described by a nucleation-dependent polymerization model.³⁴ The kinetics curve can be separated into three phases: lag phase, growth phase, and plateau phase (Figure 1). The main

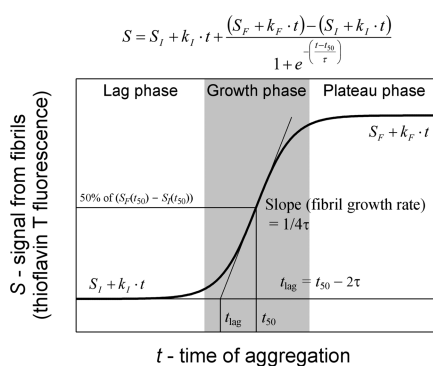


Figure 1. Typical sigmoidal aggregation curve with three aggregation phases and illustration of fitting parameters. The aggregation kinetics is described using the sigmoidal function shown at the top.

event in the lag phase is the formation of fibril nuclei. For this reason, the lag phase is often called the nucleation phase. When the fibril nuclei accumulate to certain threshold concentrations, elongation of the fibril nuclei becomes the dominant event, leading to the fibril growth phase. Eventually, the $A\beta$ monomers are largely exhausted and fibril growth reaches a plateau. There are three parameters that may represent some aspects of aggregation rate: lag time, half-time, and apparent fibril growth rate in the growth phase (Figure 1). These three parameters are also related, as shown in Figure 1. Lag time is closely related to the nucleation rate of $A\beta$ fibrils, although secondary nucleation may complicate the exact meaning of lag time.³⁵ The amplitude of the plateau represents the amount of $A\beta$ fibrils at the end of the kinetics experiment.

The thioflavin T fluorescence amplitude was used to evaluate the amount of $A\beta$ 42 fibrils.³⁶ The kinetics data were

fitted to a sigmoidal function to obtain the lag time, half-time, and apparent fibril growth rate. We found that EGCG reduces the yield of $A\beta$ 42 aggregation in a concentration-dependent manner, consistent with previous findings. The amyloid-reducing effect was confirmed using electron paramagnetic resonance (EPR) experiments. The effect on the rate of $A\beta$ 42 aggregation, however, is complicated. While low concentrations of EGCG delayed the formation of $A\beta$ 42 fibrils, high concentrations of EGCG promoted fibril formation. The implication of our findings in the context of designing human interventions is discussed.

RESULTS AND DISCUSSION

Effect of EGCG on the Rate of $A\beta$ 42 Aggregation.

Using a recombinant system to produce $A\beta$ proteins, we have demonstrated the capability to generate aggregation kinetics data with well-resolved lag phase.^{37–39} This allows us to study the effect of EGCG on the rate of $A\beta$ 42 aggregation. We performed an $A\beta$ 42 aggregation at a 2 μ M concentration, with EGCG concentrations at 0, 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, and 40 μ M, representing EGCG-to- $A\beta$ 42 ratios of 0:1, 0.05:1, 0.1:1, 0.2:1, 0.5:1, 1:1, 2:1, 5:1, 10:1, and 20:1, respectively. All of the different EGCG-to- $A\beta$ 42 ratios were performed in a single experiment, starting from a master mix containing $A\beta$ 42 at 2.2 μ M and thioflavin T at 22 μ M in phosphate-buffered saline (PBS). Separately, we made a series of EGCG stock solutions at 1–400 μ M, using a serial dilution from 400 μ M EGCG. Then, 45 μ L of the master mix was mixed with 5 μ L of the EGCG stock at different concentrations. With this experimental setup, the $A\beta$ 42 and thioflavin T were identical in each aggregation reaction, making any differences attributable to EGCG concentrations. Because we used a serial dilution to make different EGCG stocks from the 400 μ M concentration, the relative EGCG concentration in different aggregation samples is also well controlled, even though the absolute EGCG concentration may be subject to errors such as due to EGCG purity.

The aggregation was performed in PBS buffer at 37°C without agitation. The kinetics traces are shown in Figure 2A. For clarity, these aggregation data are displayed in two figure panels. Qualitatively, the aggregation traces are shifted to the left at EGCG-to- $A\beta$ 42 ratios of 1:1 or higher, suggesting accelerated aggregation (Figure 2A, top panel). At EGCG-to- $A\beta$ 42 ratios of lower than 1:1, the aggregation traces are shifted to the right, suggesting delayed aggregation (Figure 2A, bottom panel). To quantify the lag time, half-time, and fibril growth rate (i.e., the slope in the growth phase), we fitted the data to a sigmoidal function (see Methods and Figure 1), and the fitted kinetics parameters are shown in Figure 2B–D. All of the data were fitted with the exception of the EGCG-to- $A\beta$ 42 ratio of 20:1, which shows very low amplitude in thioflavin T fluorescence and the fitting did not converge. All of the fitted traces are shown in Supporting Information Figure S1.

At the low end of EGCG-to- $A\beta$ 42 ratios (i.e., 0.05:1), the lag time is longer than that of $A\beta$ 42 without EGCG (Figure 2B), suggesting that small amount of EGCG delayed $A\beta$ 42 aggregation. However, the lag time decreases with increasing concentrations of EGCG from 0.05 to 2 μ M, suggesting that higher EGCG-to- $A\beta$ 42 ratios promote $A\beta$ 42 aggregation. The aggregation-promoting effect of EGCG appears to have a limit because no dramatic changes in the lag time were observed when further increasing EGCG concentration from 2 to 20 μ M. It is also worth noting that the lag time was not

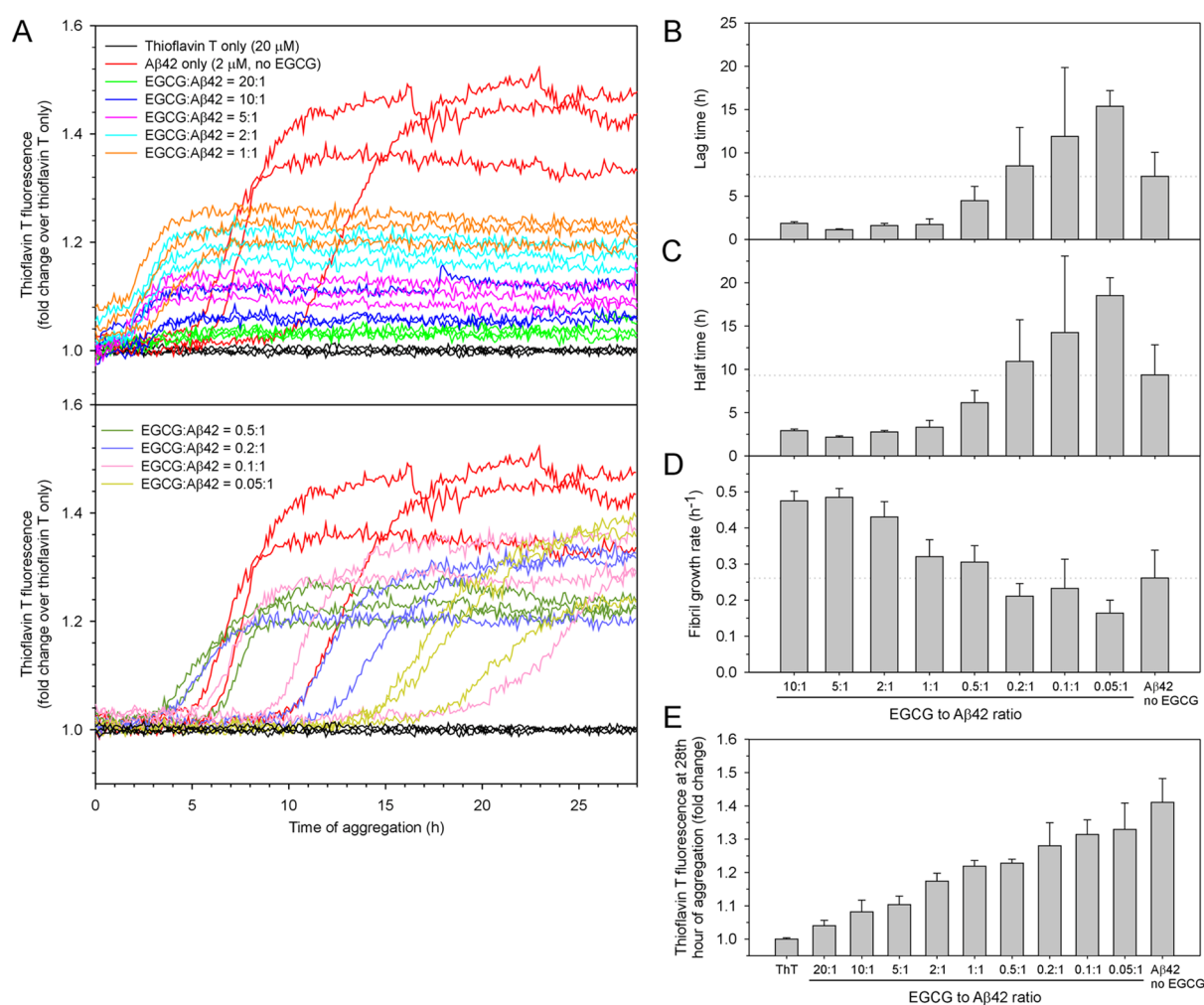


Figure 2. Effect of EGCG on the rate and yield of $A\beta_{42}$ aggregation. (A) Aggregation of $2 \mu\text{M}$ $A\beta_{42}$ in the absence and presence of 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, and $40 \mu\text{M}$ of EGCG, representing EGCG-to- $A\beta_{42}$ ratios of 0.05:1, 0.1:1, 0.2:1, 0.5:1, 1:1, 2:1, 5:1, 10:1, and 20:1. The aggregation was performed in PBS buffer at 37°C without agitation. Three replicates were performed for all samples. (B–D) Lag time, half-time, and fibril growth rate were obtained by fitting the aggregation data to a sigmoidal function (see Figure 1 and Methods). (E) Fluorescence intensity in the 27th hour of aggregation is plotted as a function of the EGCG-to- $A\beta_{42}$ ratio. Columns are the mean and error bars are the standard deviation from the three aggregation repeats.

completely eliminated even when EGCG-to- $A\beta_{42}$ ratios reach 10:1. The half-time data show a very similar trend (Figure 2C), which is not surprising because the lag time and the half-time are closely related (Figure 1).

The slope of the fibril growth phase represents the apparent fibril growth rate. Our data in Figure 2D shows that the fibril growth rate is higher with high EGCG concentrations (EGCG-to- $A\beta_{42}$ ratios of 10:1, 5:1, and 2:1) than those with low EGCG concentrations (EGCG-to- $A\beta_{42}$ ratios of 1:1 and lower). This is consistent with the accelerating effect of EGCG on the lag time and half-time at high concentrations, as shown in Figure 2B,C.

Based on the analysis of lag time, half-time, and fibril growth rate, the effect of EGCG on the $A\beta_{42}$ aggregation rate is complex. High EGCG concentrations appear to accelerate $A\beta_{42}$ aggregation, but the effect is limited as increasing the EGCG ratio beyond 1:1 and up to 10:1 did not completely eliminate the lag phase. Lower EGCG ratios of 0.2:1, 0.1:1, and 0.05:1 slowed down the $A\beta_{42}$ aggregation by increasing the lag time.

Effect of EGCG on the Yield of $A\beta_{42}$ Aggregation. In addition to the rate of aggregation, EGCG also showed a concentration-dependent effect on the amplitude of thioflavin T fluorescence (Figure 2A). To quantify this effect, we averaged the fluorescence readings in the last hour of aggregation and plotted them as a function of EGCG concentration (Figure 2E). The results show a monotonic decline in thioflavin T fluorescence with increasing concentrations of EGCG. Thioflavin T fluorescence can be affected by a number of factors. Fibril structure determines thioflavin T binding affinity and its fluorescence quantum yield. The concentration of thioflavin T and the presence of competing binders can also affect the number of thioflavin T molecules bound to the fibril. Xue et al. showed that $A\beta_{42}$ fibril amount scales linearly with thioflavin T fluorescence for the same type of fibril at a fixed thioflavin T concentration.³⁶ With the assumption that EGCG does not change the fibril structure or interfere with thioflavin T binding to a great extent, we can conclude that EGCG reduces the yield of $A\beta_{42}$ aggregation in a concentration-dependent manner. This is consistent with several previous investigations^{27–30} in which EGCG was

shown to reduce the thioflavin T fluorescence of $A\beta$ 42 aggregation, and these studies^{27–30} also concluded that EGCG inhibited $A\beta$ aggregation.

The main evidence supporting that EGCG reduces $A\beta$ 42 aggregation yield came from TEM studies, which showed amorphous³¹ or globular aggregates^{29,30} in the presence of EGCG. In this work, we performed TEM studies to examine the morphology of the $A\beta$ 42 aggregates. As shown in Figure 3,

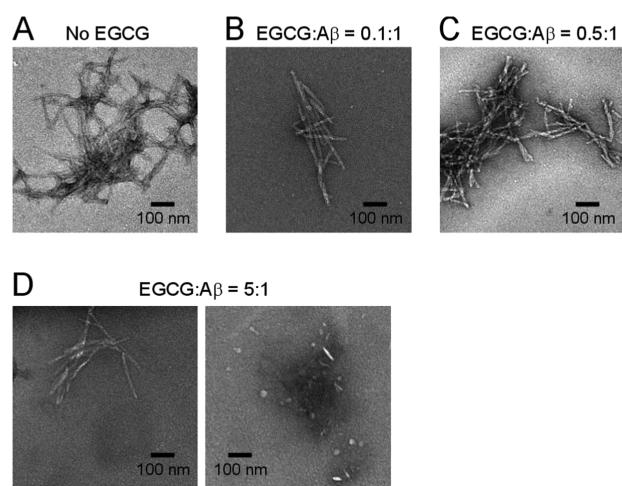


Figure 3. Transmission electron microscopy images of $A\beta$ 42 aggregates in the absence (A) and presence of EGCG at different ratios (B–D). Note that at an EGCG ratio of 5:1, both fibrillar and globular oligomers were observed.

fibrils are dominant in the low ratios (0.1:1 and 0.5:1) of EGCG to $A\beta$ 42, similar to those of the no-EGCG sample. In the EGCG-to- $A\beta$ 42 ratio of 5:1 sample, we observed both fibrillar and nonfibrillar aggregates (Figure 3D). Ehrnhoefer et al.³⁰ suggested that EGCG redirects $A\beta$ to form off-pathway oligomers. Our results are consistent with such an explanation. Unfortunately, TEM is not a quantitative technique to assess the concentration-dependent effect of EGCG on the yield of $A\beta$ aggregation because different aggregates may have different binding and staining properties on EM grids. This motivated us to further study EGCG's effect on $A\beta$ aggregation yield using spin labeling and EPR spectroscopy.

Although the decrease in thioflavin T fluorescence likely results from the reduction of aggregation yield by EGCG, it is possible that EGCG simply interfered with the binding between thioflavin T and $A\beta$ 42 fibrils without actually affecting the aggregation yield. Palhano et al.³³ showed that EGCG interfered, but did not abolish, thioflavin T binding to $A\beta$ 40 fibrils. Using saturation transfer difference NMR, Roy et al.⁴⁰ showed that EGCG competes with thioflavin T for the same binding sites on α -synuclein fibrils. Therefore, the explanation for the reduction in thioflavin T fluorescence may be complicated. To further investigate this, we assessed the effect of EGCG on the yield of $A\beta$ 42 aggregation using EPR spectroscopy. We used $A\beta$ 42 with a spin label named R1 introduced at residue Leu-34 to prepare fibrils in the absence and presence of EGCG with EGCG-to- $A\beta$ 42 ratios of 10:1 and 50:1 (Figure 4A). Aggregation kinetics shows that EGCG at these ratios reduced the thioflavin T fluorescence to near-background levels (Supporting Information Figure S2). The three samples have the same amount of $A\beta$ 42 at the beginning of aggregation. At the end of aggregation, all insoluble

aggregates were collected by centrifugation and loaded into EPR capillaries. The parameters for EPR measurements were kept the same for the three samples to allow quantitative comparison of the insoluble aggregates. The EPR spectra of these three samples are shown in Figure 4B. There is a clear difference in the overall amplitude of the EPR spectra, suggesting that EGCG reduced the amount of $A\beta$ 42 fibrils in a concentration-dependent manner. We quantified the number of spins in the three samples by calculating the double integral of the EPR spectrum. The results show that the amount of fibrils in the 10:1 (EGCG: $A\beta$ 42) sample is 36% of the no-EGCG sample, and the fibril amount is reduced to 20% in the 50:1 (EGCG: $A\beta$ 42) sample.

The EPR spectrum of $A\beta$ 42 L34R1 fibrils in the absence of EGCG is characteristic of the parallel in-register β -sheet structure.^{41–43} The EPR spectra in the presence of 10 \times and 50 \times EGCGs have a similar line shape compared to those of the no-EGCG sample (Figure 4C), suggesting that EGCG did not change the structure of $A\beta$ 42 fibrils. Previously, we have obtained the EPR spectra of $A\beta$ 42 globulomers⁴⁴ (Figure 4D) and prefibrillar oligomers⁴⁵ (Figure 4E) spin-labeled at the same residue position. The EPR spectra of these oligomers are reproduced here for comparison to show the distinct spectral line shape between oligomers and fibrils.

The rate and yield of aggregation normally have a positive correlation. The most straightforward example is probably the aggregation of the same protein under identical conditions but with different concentrations. At lower concentrations, the rate of aggregation is slower, and the yield of aggregation is also lower. In contrast, at higher concentrations, the rate of aggregation is faster, and there are more aggregates formed. Numerous examples for this exist in the literature, including $A\beta$,⁴⁶ α -synuclein,⁴⁷ and tau.⁴⁷ This issue gets complicated when the aggregation is used to investigate the effect of mutations or aggregation-modifying compounds. For example, in the alanine scanning mutagenesis study of $A\beta$ 40, Williams et al.⁴⁸ showed that $A\beta$ 40 K16A mutant had a slower rate than E22A, but a higher thioflavin T fluorescence intensity at aggregation plateau. In a spin label scanning study of $A\beta$ 42 in which the aggregation kinetics of 42 spin-labeled $A\beta$ 42 was analyzed, Hsu et al.³⁷ found that there was no correlation between the half-time of aggregation and thioflavin T fluorescence amplitude. Although inhibiting aggregation can mean either slowing down aggregation rate or reducing aggregation yield, it is important to make the distinction because the rate and yield may not change at the same direction.

Our findings that EGCG promotes the rate of $A\beta$ 42 aggregation at high EGCG-to- $A\beta$ ratios while at the same time reduces the amount of $A\beta$ 42 fibrils have important implications for using EGCG as a potential treatment or prevention for Alzheimer's disease. If the goal is to reduce the amyloid load, it is clear that higher concentrations of EGCG have a more pronounced effect. Therefore, it is desirable to use the highest dosage that is safely achievable. At the time of diagnosis, patients with Alzheimer's disease already have a large amount of amyloid deposits in the brain. At this stage, one main therapeutic goal is to reduce amyloid load, so high concentrations of EGCG are desired. If the goal is to delay the appearance of amyloid plaques, however, caution should be taken to investigate the effect of EGCG on $A\beta$ 42 aggregation at physiologically relevant concentrations. Our results show that low concentrations of EGCG delayed $A\beta$ 42 aggregation,

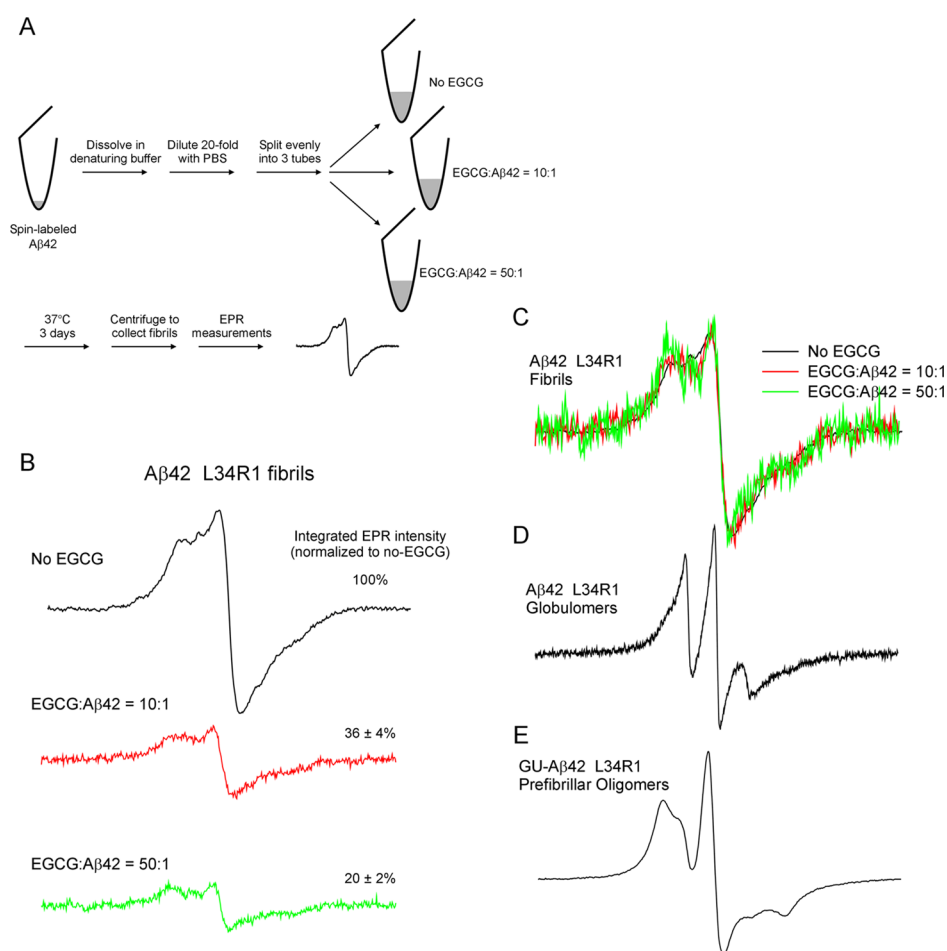


Figure 4. EPR studies of spin-labeled $A\beta_{42}$ fibrils formed in the absence and presence of EGCG. (A) Schematic diagram showing the experimental procedure. (B) EPR spectra of $A\beta_{42}$ fibrils spin-labeled at residue L34. R1 represents the spin label and is introduced by site-directed spin labeling. (C) EPR spectra in panel B are normalized to the same centerline amplitude to allow comparison of the EPR line shape. Note that the EPR spectra in the presence of EGCG show similar line shape as the no-EGCG sample, indicating similar fibril structure. (D) EPR spectrum of $A\beta_{42}$ L34R1 globulomers, reproduced using data in Gu et al.⁴⁴ (E) EPR spectrum of $A\beta_{42}$ L34R1 prefibrillar oligomers, reproduced using data in Gu et al.⁴⁵ The scan width is 200 G for all of the EPR spectra.

while higher concentrations of EGCG promoted $A\beta_{42}$ aggregation (Figure 2). It is important to uncover this EGCG concentration-dependent effect at physiologically relevant $A\beta$ concentrations. There are reports that EGCG reduces the amount of amyloids in transgenic mice, but the authors attribute the effect to the α -secretase pathway.^{23,26} The effect of EGCG on the rate of amyloid formation in transgenic animals has not yet been investigated. *In vitro*, concentrations in the picomolar and nanomolar ranges can be studied using highly sensitive fluorescence methods,⁴⁹ which may provide further insights into the interactions between $A\beta$ and EGCG.

Our findings also have implications on the mechanism by which EGCG affects $A\beta$ aggregation (Figure 5). Previously, it has been observed that EGCG redirects $A\beta_{42}$ aggregation toward off-pathway oligomerization.³⁰ This effect can, in essence, reduce the effective $A\beta_{42}$ concentration and thus leads to a reduced aggregation rate. Furthermore, EGCG can also remodel preformed $A\beta_{42}$ fibrils.³² This effect can reduce the rate of secondary nucleation, which is catalyzed by fibrillar aggregates.⁵⁰ Perhaps, more surprising is the increased aggregation rate at high EGCG-to- $A\beta$ ratios (Figure 2). We speculate that this is due to the reversibility of the EGCG's remodeling effect on $A\beta$ fibrils (shown as a red arrow in the

schematic illustration in Figure 5). In other words, we think that the EGCG-induced oligomers can also form fibrils, but the EGCG-induced oligomers are much more stable than fibrils, thus leading to a net reduction of the amount of fibril. The rate of the fibril formation, however, may be accelerated by EGCG when EGCG-induced oligomers accumulate to high concentrations.

In recent years, sophisticated kinetic analysis has greatly improved our understanding of the $A\beta$ aggregation process. Notably, the concept of secondary nucleation⁵⁰ has led to breakthrough investigations into the microscopic steps of the $A\beta$ aggregation process.^{51–53} Kinetic data have also been used to estimate the size of the nuclei in both primary and secondary nucleation.^{54,55} Knowles and colleagues developed an online platform called AmyloFit that allows the testing of various aggregation models by nonexpert users.⁵⁶ A range of aggregation mechanisms including secondary nucleation, fragmentation, and saturation can be tested using AmyloFit. Furthermore, global fitting of the kinetic parameters can be used to investigate the specific microscopic steps of the aggregation pathway. For aggregation inhibitors such as EGCG, AmyloFit can be used to provide mechanistic insights into the action of EGCG on $A\beta$ aggregation. It has been

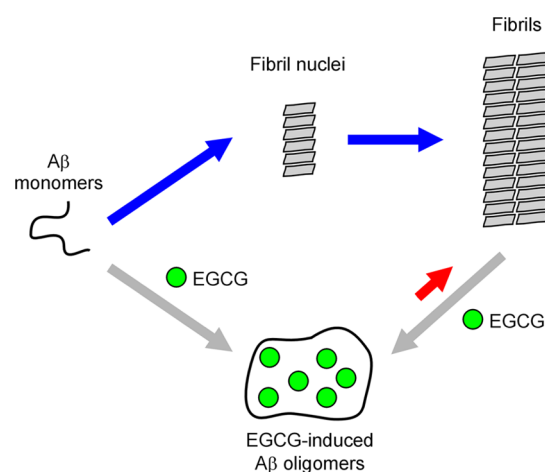


Figure 5. Schematic illustration of $A\beta$ aggregation showing a potential mechanism of action by EGCG. A simplified nucleation-dependent polymerization mechanism for $A\beta$ aggregation is depicted. EGCG can induce the formation of oligomers from $A\beta$ monomers, leading to a reduction of the effective $A\beta$ monomer concentration. EGCG can also remodel $A\beta$ fibrils to form oligomers, thus decreasing the fibril-catalyzed secondary nucleation. We speculate that EGCG-induced oligomers can also form fibrils, as represented by the red arrow. In the cases of high EGCG-to- $A\beta$ ratios, this aggregation pathway may significantly contribute to the overall aggregation rate. If the aggregation rate from EGCG-induced oligomers (red arrow) is faster than the spontaneous $A\beta$ aggregation rate (blue arrows), EGCG would show a net acceleration effect for the rate of $A\beta$ aggregation.

previously shown that $A\beta_{42}$ aggregates via a secondary nucleation mechanism.⁵⁰ As a preliminary analysis, we investigated whether the effect of EGCG in our studies can be explained by the action on either primary or secondary nucleation processes. In AmyloFit, we either fit secondary nucleation globally and primary nucleation individually (Figure S4A) or fit primary nucleation globally and secondary nucleation individually (Figure S4B). We found that neither approach led to good fits for all of the aggregation data across the full range of EGCG concentration (Figure S4). As pointed out by Meisl et al.,⁵⁶ this means that the action of EGCG on $A\beta$ aggregation is complex and may involve different microscopic processes at different EGCG concentrations. EGCG at high concentrations may mainly affect primary nucleation, while EGCG at low concentrations may primarily affect secondary nucleation, and this is consistent with our proposed model in Figure 5. $A\beta$ aggregation is a complicated system, which may include primary nucleation, secondary nucleation, fibril fragmentation, and nucleated conformational conversion. In addition to the more common mechanism of monomer addition, oligomer addition was also proposed as a mechanism of fibril growth.^{57,58} To conclusively investigate these microscopic steps using aggregation kinetics, future studies of $A\beta$ aggregation at a broad range of $A\beta$ and EGCG concentrations will be needed to delineate the detailed mechanism of interactions between EGCG and various $A\beta$ -aggregated species.

METHODS

Preparation of $A\beta_{42}$ Proteins. $A\beta$ protein was expressed in *Escherichia coli* as a fusion protein, GroES-ubiquitin- $A\beta$, as described previously.^{36,38} The fusion protein partners were then cleaved with a deubiquitylating enzyme to obtain full-

length $A\beta$ without any extra residues.⁵⁹ Afterward, $A\beta$ was buffer-exchanged to 30 mM ammonium acetate (pH 10.0) and lyophilized in small-volume aliquots. The lyophilized $A\beta$ powder was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a 100 μ M concentration with shaking (1000 rpm) at room temperature for 24 h. The HFIP was dried overnight in a chemical hood and the HFIP-treated $A\beta$ film was stored at -80°C .

Aggregation Kinetics of $A\beta_{42}$ in the Absence and Presence of EGCG. One tube of HFIP-treated $A\beta_{42}$ film was dissolved in 1 mL of a high-pH denaturing buffer called CG (20 mM CAPS, 7 M guanidine hydrochloride, pH 11) and bath-sonicated for 5 min. Then, the sample was buffer-exchanged to PBS (50 mM phosphate, 140 mM NaCl, pH 7.4) using a 5 mL HiTrap desalting column (GE Healthcare). Afterward, the sample was filtered through a 100 kD ultrafiltration filter (Sartorius Vivaspin 500) and was referred to as 100 kD filtrate. The concentration of $A\beta_{42}$ was determined to be 2.7 μ M using the fluorescamine method (see below). A fresh thioflavin T (Sigma-Aldrich, catalog #T3516) solution was made in PBS, and the concentration was determined using the extinction coefficient of $36\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 412 nm.³⁶ Then, we made a master mix containing 2.2 μ M $A\beta_{42}$ and 22 μ M thioflavin T in PBS. The purpose of the master mix is to ensure that $A\beta_{42}$ and thioflavin T are identical in all of the aggregation reactions and any differences can be attributed to EGCG concentrations.

A stock solution of EGCG (Tocris Bioscience, catalog #4524) was made at 400 μ M in water using the weight of EGCG. The EGCG stock was then serially diluted to concentrations of 200, 100, 40, 20, 10, 4, 2, and 1 μ M. To set up aggregation, 45 μ L of master mix was mixed with 5 μ L of EGCG stock solutions at different concentrations or 5 μ L of water for the no-EGCG sample. There are three replicates for each aggregation condition. Finally, all 50 μ L of the aggregation mix was transferred to a black 384-well nonbinding surface microplate with a clear bottom (Corning, catalog #3655) and sealed with a polyester-based sealing film (Corning, catalog #PCR-SP). All of the mixing steps were performed on ice. The fibrillization was started by incubating the plate in a Victor 3V plate reader (Perkin Elmer) at 37°C without agitation. The thioflavin T fluorescence was measured from the bottom of the plate with an excitation filter of 450 nm and an emission filter of 490 nm. We reported the aggregation data as fold change in fluorescence intensity by dividing the average of thioflavin T-alone sample at each time of measurements. We would like to point out that the fold change in thioflavin T fluorescence as a result of $A\beta$ aggregation may vary greatly when different fluorescence readers are used. This is likely due to the type of light source, measurement settings, detector sensitivity, and other instrument-specific factors of the plate reader, which gives various levels of background readings.

Measurement of $A\beta$ Concentration Using the Fluorescamine Method. Because the $A\beta_{42}$ concentration is low and could not be reliably determined using absorbance at 280 nm, we have developed a fluorescamine method that allows the accurate measurement of $A\beta$ concentration with sub-micromolar sensitivity.⁶⁰ The standard assay mix contains 40 μ L of PBS, 5 μ L of sample, and 5 μ L of 5 mM fluorescamine (Acros Organics, pure grade, catalog #AC19167) dissolved in acetonitrile. Upon mixing, the sample is transferred to a black 384-well microplate (Corning, catalog #3655). The

fluorescence measurement was taken using a Gemini EM plate reader (Molecular Devices) with excitation at 390 nm and emission at 478 nm. A standard curve was prepared using hen egg white lysozyme (Fisher Bioreagents, crystalline powder, catalog #BP535) with a concentration range of 0.1–1 μM , corresponding to amine concentrations of 0.7–7 μM . $A\beta$ was diluted if needed so that the fluorescence reading falls in the middle range of the standard curve.

Analysis of Aggregation Data. The aggregation data were fitted to the equation below, which was based on the work of Nielsen et al.⁶¹

$$S = S_1 + k_1 \cdot t + \frac{(S_F + k_F \cdot t) - (S_1 + k_1 \cdot t)}{1 + e^{-(t-t_{50})/\tau}}$$

Here S is the fluorescence intensity, t is the aggregation time, t_{50} (half-time) is the time to reach 50% of the maximum fluorescence, and τ is the time it takes for the fluorescence signal to change from 26.9 to 50%, or from 50 to 73.1% of the maximum fluorescence. The change in fluorescence in the lag phase is described by $S_1 + k_1 \cdot t$, and the fluorescence in the plateau phase is described by $S_F + k_F \cdot t$. The lag time is given by $t_{50} - 2\tau$, and the maximum fibril growth rate at t_{50} is given by $1/4\tau$. All of the fits are shown in Supporting Information Figure S1.

Fitting of the aggregation data using AmyloFit was performed as previously described by Meisl et al.⁵⁶ using the unseeded secondary nucleation-dominated model. The fitting results are shown in Supporting Information Figure S3. The secondary nucleation model and associated fitting parameters have been described in detail in Cohen et al.⁵⁰ and Meisl et al.⁶²

Transmission Electron Microscopy. For transmission electron microscopy, 5 μL of the aggregation sample described above after approximately 48 h of incubation was applied to glow-discharged copper grids (400 mesh formvar/carbon film, Ted Pella, catalog #01754-F) and stained with 2% uranyl acetate. The grids were then examined under an FEI T12 electron microscope with an accelerating voltage of 120 kV.

Site-Directed Spin Labeling. First, the residue L34 was mutated to a cysteine using site-directed mutagenesis. Then, $A\beta$ 42 L34C was purified and fusion partners were cleaved as described above. To attach the spin label R1, the $A\beta$ 42 L34C protein was incubated with the spin labeling reagent MTSSL (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate, AdipoGen Life Sciences) at 5-fold molar excess at room temperature for 1 h. A buffer exchange was then performed to remove the free spin label and change the buffer to 30 mM ammonium acetate (pH 10) for lyophilization. Lyophilized $A\beta$ 42 L34R1 was treated with HFIP as described above and stored at -80°C .

Preparation of Spin-Labeled $A\beta$ 42 Fibrils in the Absence and Presence of EGCG. One tube of HFIP-treated $A\beta$ 42 L34R1 protein was dissolved in CG buffer to a final concentration of 400 μM , which was determined using absorbance at 280 nm. Then, 25 μL of the $A\beta$ 42 sample in CG buffer was mixed with 475 μL of PBS to obtain the no-EGCG sample. Two other $A\beta$ 42 samples at 25 μL volume were mixed with 475 μL of PBS containing 200 μM and 1 mM EGCG to obtain the EGCG-to- $A\beta$ 42 ratio of 10:1 and 50:1, respectively. These three samples were incubated at 37 $^\circ\text{C}$ for 3 days without agitation. At the end of incubation, the three samples were centrifuged at 20 000g for 20 min to pellet the $A\beta$ 42

aggregates. The pellet was surface-washed twice with PBS to remove loosely associated $A\beta$ 42 proteins.

EPR Spectroscopy. The pellets of $A\beta$ 42 L34R1 after centrifugation were resuspended in 15 μL of PBS and then loaded in glass capillary tubes (VitroCom) sealed at one end. EPR spectra were collected at X-band using a Bruker EMXnano spectrometer at room temperature using a microwave power of 15 mW. A modulation frequency of 100 kHz was used, and the modulation amplitude was set at 4 G based on the line width of the EPR spectrum. The scan width was 200 G. The same parameters for data acquisition, including the number of scans, were used for all three $A\beta$ 42 samples prepared with or without EGCG. To quantify the amount of spin-labeled aggregates from the EPR spectra, double integrals were calculated using an in-house program written by Dr. Christian Altenbach at the University of California, Los Angeles. Errors were estimated by adjusting the parameters for baseline correction to obtain the lower and upper ranges of the calculated double integrals. The double integrals were then normalized as a percentage of the no-EGCG sample, which is set at 100%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c02063>.

Sigmoidal fits for fibrillization kinetics of $A\beta$ 42 samples in the absence and presence of EGCG (Figure S1); aggregation of $A\beta$ 42 spin-labeled at position 34 in the absence and presence of EGCG (Figure S2); and fitting of aggregation data using AmyloFit (Figure S3) (PDF)

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Author Contributions

G.P., C.X., and H.W. designed and carried out experiments, analyzed data, and drafted the manuscript. Z.G. conceived and supervised the study, designed the experiments, and drafted the manuscript. All authors gave final approval for publication.

Notes

The authors declare no competing financial interest.

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