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Curcumin Attenuates Amyloid-β Aggregate Toxicity and Modulates Amyloid-β Aggregation Pathway

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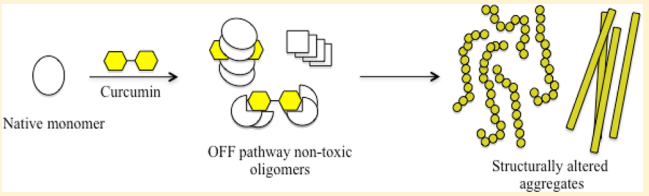
Supporting Information

ABSTRACT: The abnormal misfolding and aggregation of amyloid-β (Aβ) peptides into β-sheet enriched insoluble deposits initiates a cascade of events leading to pathological processes and culminating in cognitive decline in Alzheimer's disease (AD). In particular, soluble oligomeric/prefibrillar Aβ have been shown to be potent neurotoxins. The naturally occurring polyphenol curcumin has been shown to exert a neuroprotective effect against age-related neurodegenerative diseases such as AD. However, its protective mechanism remains unclear. In this study, we investigated the effects of curcumin on the aggregation of Aβ40 as well as Aβ40 aggregate induced neurotoxicity. Our results show that the curcumin does not inhibit Aβ fibril formation, but rather enriches the population of "off-pathway" soluble oligomers and prefibrillar aggregates that were nontoxic. Curcumin also exerted a nonspecific neuroprotective effect, reducing toxicities induced by a range of Aβ conformers, including monomeric, oligomeric, prefibrillar, and fibrillar Aβ. The neuroprotective effect is possibly membrane-mediated, as curcumin reduced the extent of cell membrane permeabilization induced by Aβ aggregates. Taken together, our study shows that curcumin exerts its neuroprotective effect against Aβ induced toxicity through at least two concerted pathways, modifying the Aβ aggregation pathway toward the formation of nontoxic aggregates and ameliorating Aβ-induced toxicity possibly through a nonspecific pathway.



KEYWORDS: *Alzheimer's disease, amyloid-β peptide (Aβ), Aβ aggregation, toxicity, curcumin, neuroprotection*

Alzheimer's disease (AD) is the most prevalent progressive neurodegenerative disorder, accounting for 60−80% of all cases of dementia.[1−3] The amyloid-β peptide (Aβ), generated through the sequential proteolysis of the transmembrane amyloid precursor protein (APP) by β- and γ-secretases, is widely believed to be directly involved in the pathogenesis of AD.[1,4] Although several isoforms exist, ranging in length from 39 to 43 residues, Aβ40 and Aβ42 are the most physiologically relevant.[5,6] In AD patients, Aβ40 is the most abundant (9:1 Aβ40: Aβ42), but Aβ42 is more aggregation-prone. Research from the past three decades have led to the current view that the abnormal misfolding and aggregation of Aβ peptides into β-sheet enriched insoluble deposits initiates a cascade of events leading to pathological processes that culminate in cognitive decline in AD.[1−4,7] Therefore, inhibiting or disrupting the Aβ aggregation process represents a promising therapeutic strategy for the prevention and treatment of AD.

The Aβ aggregation process is highly complex, involving multiple pathways (on, off, or double-concerted pathways) that generate wide-ranging Aβ aggregates.[8−11] In vitro, Aβ has been found to aggregate through both template-dependent (heterogeneous) as well as independent (homogeneous) mechanisms.[10,11] During aggregation, Aβ monomers undergo conformational changes to form misfolded intermediates that subsequently assemble into β-sheets enriched conformers, including oligomers, protofibrils, and eventually fibrils.[12,13] Aβ dimers, trimers, and a range of other soluble oligomers have been found in sodium dodecyl sulfate (SDS)-soluble fraction of AD brain extracts.[14−17] One particular toxic oligomer, dodecameric Aβ*56, has been isolated and identified from Tg2576 mice with memory deficits.[18] Toxic small globular oligomers (∼5 nm in diameter), also known as Aβ-derived diffusible ligands (ADDLs), have also been detected in AD brains.[19,20] Moreover, large globular/spheroidal oligomers measuring ∼10−15 nm in diameter prepared in vitro have been found to be potent neurotoxins.[21] These accumulating reports of toxic Aβ oligomers, combined with the finding that insoluble fibrillar Aβ deposits poorly correlate with clinical symptoms,[22] suggest that soluble Aβ aggregates are the primary toxic species in AD as opposed to mature Aβ fibrils.[17,23] Aβ oligomers have been found to elicit a range of cellular responses including increased calcium ion flux, altered kinase, phosphatase, and caspase activities, disrupted regulation of transcription factors, and changes in ion channel and receptor gene expression.[24−28]

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Small molecules that intercede with Aβ aggregation and/or neurotoxicity have been studied and reported for some time.[29−32] Among them, naturally occurring polyphenols, abundant in teas, berries, fruits, spices, and plants, have been extensively studied.[24,25,33−38] Aside from their well-known antioxidative and anti-inflammatory properties, these compounds have also shown therapeutic potential in the treatment of neurodegenerative disorders, including AD.[24−26,39−42] In particular, the high level of dietary intake of the biphenolic compound curcumin, the main active component of the spice turmeric, has been suggested to account for the lower incidence and prevalence of AD in East Asian populations.[40,43,44] Several studies have shown that curcumin enhanced spatial learning and memory in an amyloid-infused AD rat.[45,46] The compound has also been found to rescue long-term potentiation in mouse hippocampus impaired by Aβ and increase the longevity in transgenic flies that express Aβ and tau proteins.[46−48] In vitro, curcumin has been found to protect against Aβ-induced death of PC-12, SH-SY5Y neuroblastoma, and human umbilical vein endothelial cells.[26,39,47,49,50]

Despite the growing number of animal and cell culture studies that demonstrate the neuroprotective effect of curcumin against age-related neurodegenerative diseases such as AD, its mechanism of action remains to be resolved. As toxic effects associated with Aβ oligomers appear to include oxidative damage and inflammation, the neuroprotective efficacy of curcumin is believed to partly derive from its antioxidative and anti-inflammatory properties. Curcumin has been found to restore homeostasis of the inflammatory system, boost the heat shock system to enhance clearance of toxic aggregates, scavenge free radicals, chelate metal ions, and induce antioxidant responses.[39−42,49] In addition, numerous studies have also examined how curcumin directly affects the Aβ aggregation process; however, inconsistent, sometimes contradictory, findings have been reported.[50−52] The dominant view is that curcumin is an aggregation inhibitor[50,51] that not only reduces the formation and extension of Aβ fibrils, but also destabilizes preformed amyloid fibrils in vitro and in vivo.[52,53] However, curcumin has also been shown to inhibit Aβ oligomerization but not fibril formation in vitro.[54] More recently, animal studies using transgenic AD mice and *Drosophila* showed that curcumin had either no effect or enhanced the formation of fibrillar deposits by reducing oligomeric/prefibrillar Aβ species.[36,48] Because small molecule modulation of Aβ aggregation and toxicity remains a very attractive therapeutic strategy and curcumin has shown exceptional promise for preventing and treating AD, determining the precise role curcumin plays in Aβ aggregation is still needed.

In this study, we investigated the effects of curcumin on Aβ aggregation as well as Aβ aggregate induced neurotoxicity. To this end, we incubated the most abundant isoform Aβ40 in the absence or presence of curcumin and characterized the range of aggregates formed using biophysical, biochemical, and cell based assays that probed aggregate morphology, secondary structures, antibody reactivity, and toxicity. These results, combined with the quantification of the soluble and insoluble aggregates formed, showed that curcumin exerts a nonspecific neuroprotective effect against Aβ aggregate induced toxicity, but does not inhibit amyloid fibril formation. On the contrary, curcumin rather enriched the population of soluble aggregates (oligomers and prefibrillar species) that are morphologically indistinguishable from toxic soluble aggregates formed in the absence of curcumin, but are nontoxic and structurally altered. This suggests that the remodeling of the Aβ aggregation process that promotes the formation of "off-pathway", nontoxic conformers underlies the observed neuroprotective effect of curcumin.

RESULTS AND DISCUSSION

Despite accumulating evidence demonstrating the neuroprotective effects of the naturally occurring polyphenol curcumin against Aβ aggregate induced toxicity in AD, its mechanism of action remains to be resolved. Aside from possessing antioxidant and anti-inflammatory properties that can prevent tissue damage,[26,41] curcumin has also been shown to reduce Aβ induced toxicity and inhibit Aβ oligomer and fibril formation.[50,52,53] However, several recent reports have also suggested that curcumin promoted Aβ fibril formation and reduced oligomeric species.[36,48,54]

Curcumin Does Not Inhibit Aβ Oligomer and Fibril Formation. In this study, we analyzed the morphologies of Aβ species formed during incubation in the presence of curcumin and compared them to those formed in the absence of curcumin. TEM images of Aβ (100 μM) incubated alone for 1 day at 37 °C showed predominately small globular species (Figure 1A1). As the size and appearance of these species were

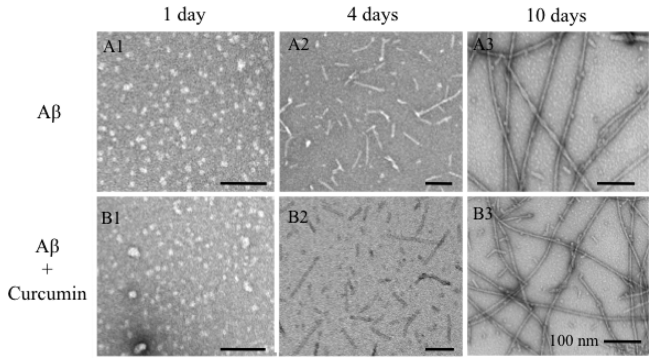

Figure 1. Transmission electron microscopy (TEM) images of 100 μM Aβ40 quiescently incubated at 37 °C for 1, 4, or 10 days in the absence (top row A1−A3) or presence (bottom row B1−B3) of 50 μM curcumin.

very similar to those seen from fresh unincubated (day 0) Aβ samples (data not shown), the 1 day incubated sample was composed primarily of monomeric Aβ. After 4 days of incubation, a mixture of larger globular oligomers and short protofibrils were visible (Figure 1A2). By day 10, mature Aβ fibrils were present (Figure 1A3). In comparison, morphologies of Aβ coincubated with curcumin (50 μM) for 1, 4, or 10 days were similar to those of Aβ incubated alone, where primarily monomeric Aβ (Figure 1B1) aggregated into larger oligomeric and protofibrillar structures (Figure 1B2) that subsequently assembled into long mature fibrils (Figure 1B3). Based on TEM images, our results thus indicate that the presence of curcumin did not inhibit Aβ40 aggregation or significantly alter the morphologies of Aβ monomers, prefibrillar or fibrillar aggregates.

In addition, we performed ThT binding assay to detect Aβ fibril formation. ThT has been hypothesized to bind to surface grooves along mature fibrils, and as a result, the dye exhibits red-shifted and enhanced fluorescence emission. As expected, Aβ incubated alone gave rise to a typical nucleation-dependent sigmoidal ThT fluorescence profile during the 10-day

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incubation period (Figure 2A, unfilled circles), with a distinct lag phase of about 4 days, a rapid fibril growth phase, and a plateau phase. In contrast, the curcumin containing Aβ samples did not give rise to significant increases in ThT fluorescence (Figure 2A, filled circles). This lack of increase of ThT fluorescence during incubation clearly is not due to the inhibition of fibril formation, as seen in TEM images (Figure 1B), but likely due to ThT quenching by curcumin or competitive fibril binding by curcumin.[55] To further investigate this phenomenon, we measured the ThT fluorescence of preformed amyloid fibrils (Aβ incubated alone for 10 days) after the addition of curcumin. Results show that ThT fluorescence rapidly decreased after the addition of curcumin (Figure 2B, filled circles), whereas in a control sample where curcumin was not added, ThT fluorescence remained unchanged (Figure 2B, unfilled circles). TEM images of the fibrils before (Figure 2C) and after the addition of curcumin (Figure 2D) also showed that the morphology of the fibrils remained unaltered by curcumin. Thus, the low ThT fluorescence of incubated Aβ samples measured in the presence of curcumin (Figure 2A, filled circles) was due to quenching and/or competitive binding, not due to the inhibition of fibril formation. Additionally, curcumin had little effect on the morphology of preformed fibrils.

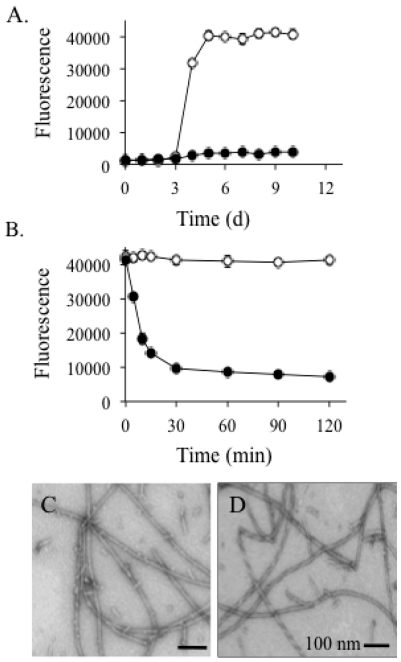

Figure 2. (A) Thioflavin-T (ThT) fluorescence profiles of 100 μM Aβ40 incubated at 37 °C for up to 12 days in the absence (unfilled circles) and presence (filled circles) of 50 μM curcumin. (B) ThT fluorescence of preformed Aβ40 fibrils (100 μM, monomer basis) after the addition of 50 μM curcumin (filled circles). Fibrils were formed by incubating Aβ samples at 37 °C for 10 days. ThT fluorescence of a control sample where curcumin was not added was also monitored (unfilled circles). (C, D) TEM images of preformed Aβ fibrils before (C) and 2 h after (D) the addition of 50 μM curcumin.

On the secondary structural level, curcumin also had very little effect on Aβ fibrils based on circular dichroism (CD) measurements (Supporting Information Figures S1 and S2). CD spectra of monomeric Aβ sample in the absence and present of curcumin overlapped and showed a maximum negative ellipticity around ~195 nm, consistent with a random coil.[12,13] Mature fibrils formed in the presence and absence of curcumin also overlapped but showed a maximum negative ellipticity at ~218 nm, indicating the presence of β-sheets. Polyphenols and flavonoids have been reported to inhibit the formation of β-sheets by Aβ.[56−58] Our results, however, show that at the secondary structural level, the presence of curcumin did not alter Aβ monomer or fibril structure.

Curcumin Reduces the Toxicity of Aβ Aggregates. Previously, we have shown that favorable interactions between monomeric Aβ40 and lipid membrane templates Aβ aggregation and concomitantly, induces membrane disruption.[59,60] We have also shown that curcumin ameliorates monomeric Aβ-induced neurotoxicity by directly attenuating Aβ-membrane interactions.[61] In this study, TEM and CD results show that curcumin did not inhibit Aβ aggregation (Figures 1, S1, and S2). Next, we sought to assess the effect of curcumin on neurotoxicity induced by the wide-ranging Aβ aggregates prepared by incubation. In each toxicity assay, cultured human neuroblastoma SH-SY5Y cells were treated with an aliquot of 10 μM Aβ or 10 μM Aβ with 5 μM curcumin and cell viability was assessed by the MTT assay, which measured cellular metabolic activity. Consistent with our previous study,[61] 10 μM of monomeric Aβ (day 0) reduced cell viability by approximately 30% (Figure 3, unfilled circles).

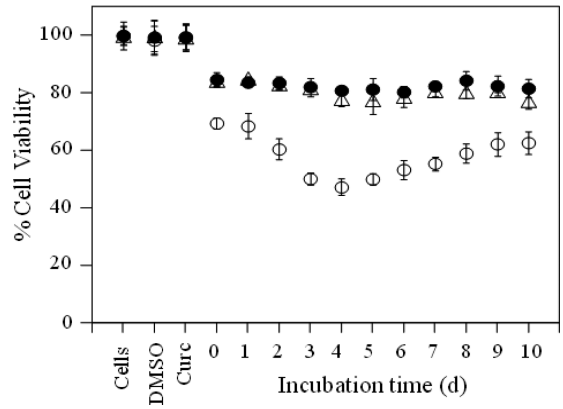

Figure 3. Viability levels of cultured SH-SY5Y cells exposed to Aβ incubated alone (unfilled circles) or coincubated with curcumin (filled circles) for up to 10 days. Cells were also pretreated with 5 μM curcumin for 3 h, rinsed, and exposed to samples of Aβ incubated alone (unfilled triangles). Incubated 100 μM Aβ samples were diluted 10 times with culture medium prior to adding to cells. Data are presented as mean ± standard deviation from three to four independent experimental measurements. The controls either received no treatment or were treated with equivalent amounts of DMSO or curcumin as those found in the cell treatment medium.

Incubation for up to 4 days further decreased cell viability, suggesting that oligomeric aggregates formed during incubation (Figure 1A2) were toxic. At longer sample incubation times, however, cell viability increased, indicating the loss of the toxic soluble oligomers and the formation of higher-order, less toxic Aβ fibrils. In comparison, cells treated with Aβ coincubated with curcumin showed higher levels of viability during the entire sample incubation period (Figure 3, filled circles). Thus, curcumin not only reduced toxicity induced by monomeric Aβ (day 0), consistent with our previous finding,[61] but coincubation also reduced the toxicity of oligomeric and fibrillar Aβ species.

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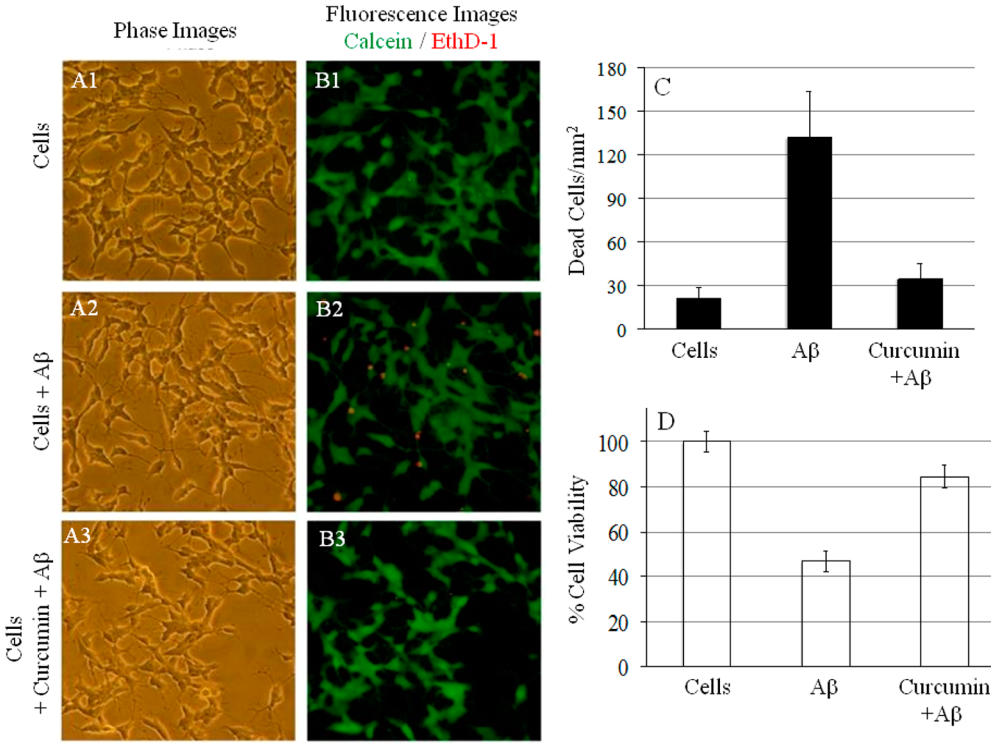

Figure 4. Live/dead staining assay results of SH-SY5Y cells exposed to Aβ aggregates. Phase images (A) and fluorescence images (B) of cultured SH-SY5Y cells alone (top images A1 and B1), exposed to 20 times diluted 100 μM Aβ incubated alone for 5 days at 37 °C (middle images A2 and B2), or pretreated with 5 μM curcumin for 3 h and then rinsed and exposed to 5-day incubated Aβ (bottom images A3 and B3). Green (calcein) fluorescent cells are live cells, and red (ethidium homodimer-1) fluorescence is from membrane compromised cells. Quantitative analysis of the live/dead assay from five to six representative images is also shown (C). MTT based viability assay of cells using the same live/dead assay conditions were also carried out and results are also shown for comparison (D). Data are presented as mean ± standard deviation from three to four independent experimental measurements.

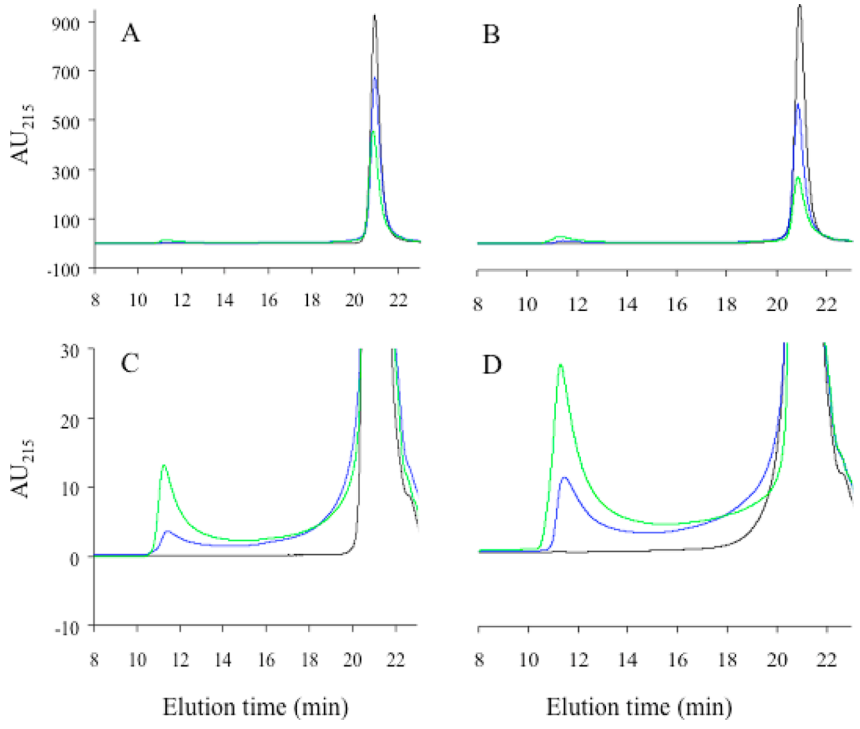

Figure 5. SE-HPLC chromatographs of Aβ incubated in the absence (A, C) or presence (B, D) of curcumin for 0 (black), 4 (blue), or 8 days (green) at 37 °C. Panels (C) and (D) are close up views of the void volume region of chromatographs in (A) and (B), respectively.

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The toxicity assay carried out as described above with samples of Aβ coincubated with curcumin exposed the cells to oligomers and fibrils formed in the presence of curcumin (imaged in Figure 1B1−B3) as well as to free curcumin in the treatment solution (5 μM). To assess whether the higher levels of cell viability were due to the presence of free curcumin or the inherently lower toxicity of the oligomers and fibrils formed in the presence of curcumin, we pretreated the cells with curcumin for 3 h, rinsed them, and added samples of Aβ incubated alone for 0 to 10 days. These curcumin pretreated cells exhibited higher levels of viability for all samples (Figure 3, unfilled triangles) compared to cells treated with the same Aβ samples (Figure 3, filled circles), indicating a nonspecific protective role of curcumin against the range of toxic Aβ conformations.

Next, to compliment the MTT metabolic activity based toxicity assay, we performed live−dead staining assay to assess whether curcumin plays a protective role against Aβ aggregate induced cell membrane disruption. In this assay, two dyes were used: the cell permeable calcein-AM, which fluoresces green due to intracellular esterase activity of viable cells, and the cell impermeable ethidium homodimer-1, which fluoresces red when it binds to nucleic acids upon entering cells with damaged membranes.[62] Thus, the cells that stain green are live cells and those that stain red are plasma membrane compromised, or dead cells. The phase (Figure 4A) and fluorescence microscopy images (Figure 4B), along with a plot of dead cell counts (Figure 4C), showed that Aβ incubated alone for 5 days caused an increase in the number of red fluorescing cells (Figure 4B2) compared to untreated cells (Figure 4B1), indicating that the Aβ sample had caused cell membrane disruption. Pretreating the cells with curcumin reduced the number of plasma membrane comprised cells (Figure 4B3), possibly due to the attenuation of Aβ-membrane interactions. Note that in these assays, cells were incubated with 5 μM of 5-day incubated Aβ (Figure 4), as opposed to the 10 μM Aβ used in the MTT assay (Figure 3). For direct comparison, a MTT assay was also carried out at the reduced Aβ concentration. As shown by our results, 5 μM of 5-day incubated Aβ significantly reduced cell viability and pretreatment with curcumin rescued cells from Aβ induced toxicity (Figure 4D). These two sets of complementary assays, one measuring cellular metabolic activity and the other probing cell membrane integrity, thus show that curcumin plays a neuroprotective role by reducing the toxicity of Aβ aggregates and/or Aβ-induced loss of cell membrane integrity.

Curcumin Accumulates Aβ Oligomers. To quantitatively determine the effect of curcumin on Aβ aggregation, we analyzed Aβ incubated alone or Aβ coincubated with curcumin by SE-HPLC. Incubated samples were first centrifuged to remove insoluble aggregates. The soluble fractions (see Methods) were then injected onto a size-exclusion column to separate different Aβ species based on size. Freshly prepared Aβ in the absence (Figure 5A) or presence of curcumin (Figure 5B) eluted as single peaks (black lines), presumably monomers, at a retention time of 20.9 min. With incubation, the monomer peak decreased (Figure 5A and B, blue (4 days incubation) and green (8 days incubation) lines), while a peak corresponding to the void volume (11.5 min retention time) appeared (zoomed in views Figure 5C and D). Proteins eluted in the void volume are large soluble species, presumably oligomers and prefibrillar species. The oligomer peak in samples of Aβ incubated alone grew with incubation time until after 7 days, after which the peak decreased, indicating the conversion of these soluble aggregates into insoluble fibrils (Figures 5C and 6B, unfilled circles). At the end of the 12-day incubation period, approximately 60% of monomeric Aβ had converted to insoluble aggregates, with 40% monomers remaining (Figure 6A and C, unfilled circles).

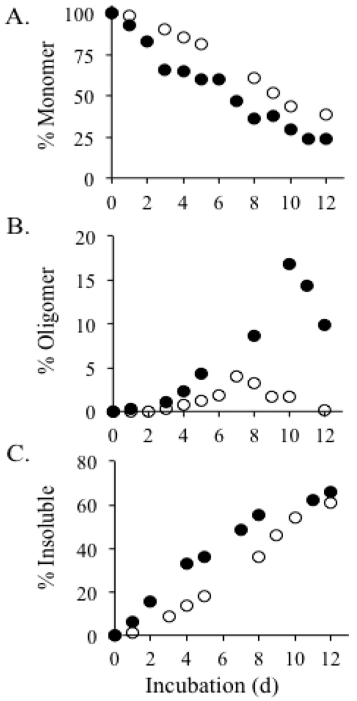

Figure 6. Profiles of monomeric (A), soluble oligomeric (B), and insoluble Aβ aggregates (C) obtained from SE-HPLC chromatogram analysis of Aβ incubated in the absence (unfilled circles) or presence (filled circles) of curcumin. Mass percentages of monomers and soluble oligomers were calculated by obtaining the areas under the peaks and then diving by the area of the monomer peak at 0 day incubation. Mass percentages of insoluble Aβ aggregates were calculated by subtracting monomer and soluble oligomer percentages from 100%.

SE-HPLC analysis of Aβ samples coincubated with curcumin gave rise to similar results, although interesting and important differences were seen. The curcumin coincubation samples gave rise to faster monomer depletion (Figures 5B and 6A, filled circles) and larger void volume peaks (Figure 5D), indicating the presence of higher levels of soluble oligomers in the samples (Figure 6B, filled circles) compared with those of Aβ incubated alone for equivalent time periods. Increases in the Aβ oligomer peak, accompanied by decreases of the monomer peak, were observed until day 10 of incubation (Figures 5D and 6B, filled circles), after which the peak decreased, presumably due to conversion into insoluble aggregates. At the end of the 12-day incubation, only about 25% of monomers remained (Figure 6A and C, filled circles), as compared to 40% of Aβ incubated alone (Figure 6A and C, unfilled circles). The amount of insoluble materials at the end of incubation, which consisted of insoluble fibrils and amorphous aggregates, accounted for ~60% of the total protein, comparable to that of Aβ incubated alone (Figure 6C). Levels of soluble aggregates (oligomers and prefibrillar aggregates) in curcumin coincubated samples were significantly higher at later incubation times (Figure 6B, filled circles). Overall, HPLC results indicate that coincubation with curcumin did not inhibit fibril formation, but

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enriched the population of soluble oligomers and prefibrillar species.

Curcumin Accumulated Oligomers Are Nontoxic Conformers. So far, our results showed that curcumin protected cells from Aβ aggregate induced toxicity (Figures 3 and 4). This effect was rather nonspecific, as the compound reduced toxicity induced by a wide range of Aβ species, including monomers, oligomers, and prefibrillar and fibrillar aggregates (Figure 3). The reduced toxicity of Aβ aggregates formed during coincubation with curcumin could be due to the presence of free curcumin in solution that the cells were exposed to (5 μM) or the effect of curcumin on Aβ aggregates; that is, aggregates formed in the presence of curcumin are nontoxic. To investigate the effect of free curcumin in solution, viability of cells pretreated with 5 μM curcumin, washed, and then exposed to toxic Aβ samples was determined. Our results demonstrated that the presence of curcumin directly attenuated Aβ toxicity (Figure 3, unfilled triangles and Figure 4).

In order to investigate the nature of the oligomers and prefibrillar species accumulated in the presence of curcumin, we isolated these soluble aggregates from 5-day coincubated samples by SE-HPLC and tested their toxicity. Note that, during the SE-HPLC separation, free curcumin in the incubation solution, that is, that not complexed or bound to Aβ oligomers, was also removed. As no additional curcumin was added during the assay, cells were not exposed to free curcumin in this assay. For comparison, Aβ oligomers formed from incubating Aβ alone for 5 days were also isolated and tested. As expected, these oligomers (~1 μM Aβ, monomer concentration) were very toxic, reducing cell viability by 50% (Figure 7A, filled bar) even at the low concentration. However, oligomers fractionated from a curcumin coincubated Aβ sample were far less toxic, reducing cell viability only by 10% (Figure 7A, unfilled bar). As an additional test, we combined the oligomer and monomer fractions from each sample and test their toxicities. The combined samples (~5 μM Aβ monomer concentration) showed the same trend as those of isolated oligomers—the sample from Aβ incubated alone induced a higher level of toxicity (Figure 7A, filled bar) compared with that from Aβ coincubated with curcumin (Figure 7A, unfilled bar).

To further distinguish the two populations of soluble Aβ aggregates, formed in the absence and presence of curcumin, we performed dot blot assay to test their reactivity toward known antibodies, both Aβ sequence specific anti-Aβ1−16 (6E10) and conformation specific (OC and A11).[54,63−65] The OC antibody recognizes generic epitope common to amyloid fibrils and fibrillar oligomers, but not random coil monomers or nonfibrillar oligomers.[64] The A11 antibody selectively recognizes epitopes common to toxic soluble oligomers of a wide variety of amyloidogenic proteins and peptides, but does not recognize either monomeric or fibrillar conformers.[54,64] Thus, used in combination, the OC and A11 antibodies can distinguish aggregated conformers that overlap in size, but differ in conformation and biological activity (i.e., toxicity). Reactivity of four different Aβ samples with the three antibodies was determined: (1) Aβ monomers, (2) Aβ oligomeric/prefibrillar species isolated from incubating the peptide alone for 5 days, (3) Aβ oligomeric/prefibrillar species isolated from coincubating the peptide with curcumin for 5 days, and (4) Aβ fibrils from incubating the peptide alone for 10 days (Figure 7B). As expected, all Aβ samples were 6E10 positive. Aβ fibrils and isolated oligomeric/prefibrillar species were OC-positive,

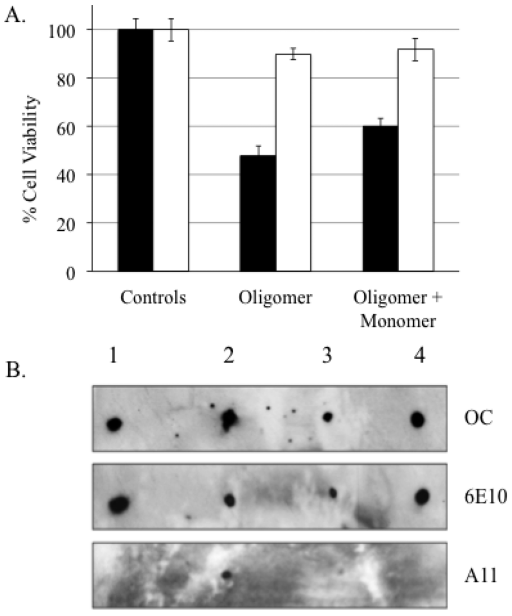

Figure 7. (A) Percent viability of SH-SY5Y cells exposed to Aβ oligomers isolated from 5-day incubated Aβ in the absence (filled bar) or presence (unfilled bar) of curcumin. Aβ oliogmers and monomers were also pooled and used for determining percent cell viability. (B) Dot blot analysis of Aβ monomers (lane 1), oligomers isolated from 5-day incubated Aβ alone (lane 2) or with curcumin (lane 3), or fibrils (lane 4). Fibrils were formed from incubating Aβ alone for 10 days. The samples were blotted onto polyvinylidene difluoride membrane and probed with anti-amyloid fibrils OC (top), anti-Aβ 6E10 (middle), and anti-oligomer A11 (bottom) antibodies.

indicating the presence of fibrillar motif in the peptide backbone. This result is reasonable as many short protofibrillar structures were seen in TEM (Figure 1A2). Surprisingly, the monomeric Aβ sample was also OC positive. This could be due to transient, fibril-like folds in the generally disordered Aβ sample; weak reactivity against Aβ monomers was also noted in the manufacturer's product overview (http://www.emdmillipore.com/). In contrast, only oligomers isolated from Aβ incubated alone for 5 days were A11 positive, indicating that these nonfibrillar aggregates share the epitope common to toxic soluble oligomers. Oligomers isolated from Aβ coincubated with curcumin for 5 days were A11 negative, as were monomeric and fibrillar Aβ species (Figure 7B). These results clearly indicate that coincubation with curcumin resulted in an enriched population of nontoxic Aβ oligomers that lack the common structural epitope shared by many toxic amyloid-soluble oligomers.

Curcumin Accumulated Oligomers Seeded the Formation of Wormlike Aβ Fibrils. To further gain insights into the differences between the two populations of oligomers, we seeded monomeric Aβ solutions (curcumin-free) with oligomers isolated from Aβ incubated alone for 5 days or those isolated from Aβ coincubated with curcumin for 5 days. ThT and TEM imaging where then used to detect fibril formation and image aggregate morphology. The oligomers isolated from coincubation with curcumin were imaged 3 h or 1 day (at 37 °C) after fractionation by HPLC and their TEM images (Figure 8A) show that these oligomers were metastable and underwent further aggregation to form longer protofibrils. We also measured ThT fluorescence profile of a monomeric Aβ sample seeded by these curcumin accumulated oligomers and

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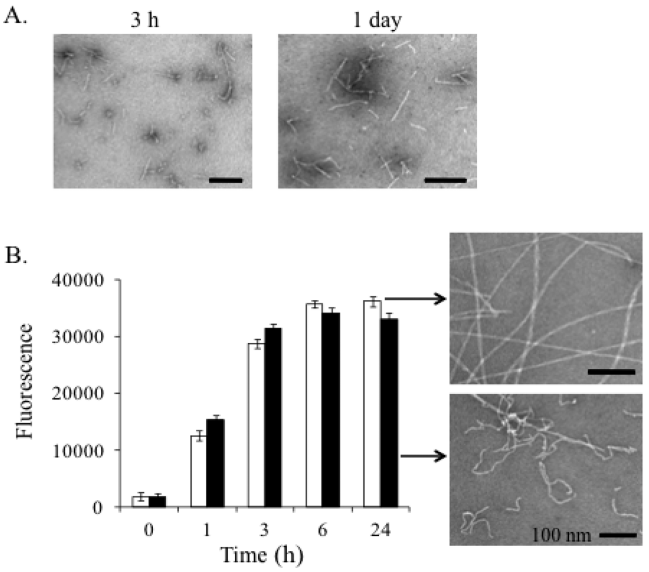

Figure 8. (A) TEM images of oligomers isolated from Aβ (100 μM) incubated in the presence of curcumin (50 μM) at 37 °C for 5 days. The images were taken after 3 h (left) or 1 day (right) (incubation at 37 °C). (B) ThT fluorescence profiles of fresh Aβ (100 μM) seeded by Aβ oligomers isolated from Aβ incubated alone for 5 days (unfilled bars) or by Aβ oligomers isolated from Aβ coincubated with curcumin for 5 days (filled bars). TEM images show morphologies of Aβ fibrils seeded by the two different populations of oligomers.

observed an immediate increase in ThT fluorescence (Figure 8B, filled bar). The elimination of lag time and observed increases in ThT fluorescence indicate that these curcumin-accumulated oligomers are capable of nucleating the formation of fibrillar aggregates. ThT fluorescence profile appeared identical to that of a monomeric Aβ sample seeded by Aβ incubated alone for 5 days (no SE-HPLC fractionation) (Figure 8B, unfilled bar). Thus, the ability of curcumin coincubated, but nontoxic, oligomers to seed further Aβ fibril formation was comparable to that of toxic oligomers from Aβ incubated alone based on ThT binding.

We next analyzed morphologies of fibrils seeded by curcumin accumulated Aβ oligomers. Strikingly, these fibrils appeared thin and wormlike (Figure 8B, bottom left panel), distinct from the straight Aβ protofibrils or fibrils typically seen from incubating Aβ alone, or even those seeded by lipid vesicles,[59,60] for example. Taken together, our results indicated that the nontoxic curcumin accumulated oligomers are structurally distinct from the toxic oligomers formed in the absence of curcumin (Figures 8 and 9). The exact structural details, however, remain to be elucidated.

Effects of Curcumin on Aβ Aggregation. Our results clearly show that curcumin does not inhibit the aggregation of the intrinsically disordered monomeric Aβ peptides into β-sheet enriched fibrillar aggregates (Figures 1, S1, and S2). Extensive aggregation occurred in Aβ samples incubated with or without curcumin. Fibrils formed under both incubation conditions were long, straight, and unbranching (Figure 1A3 and B3) and were enriched in β-sheet structures (Figure S2). As the presence of curcumin interfered with ThT fluorescence assay due to quenching and/or competitive fibril binding (Figure 2), we were unable to obtain a reliable fibrillation profile for Aβ coincubated with curcumin. However, based on monomer depletion and soluble and insoluble aggregate formation profiles obtained from SE-HPLC analysis, the rates of Aβ aggregation in the absence or presence of curcumin were comparable, with samples containing curcumin forming higher levels of soluble and insoluble aggregates during the incubation period (Figure 6).

Moreover, our results show that fibrillation of Aβ in the absence or presence of curcumin proceeded through the formation of oligomeric and prefibrillar intermediates that were morphologically similar. TEM images of 4-day incubated samples show a mixture of round/globular oligomers and short, straight prefibrillar species (Figure 1). These soluble intermediates spanned a large size range, including a significant population with molecular weights 700 kDa or higher as the aggregates eluted in the void volume of the size-exclusion column (Figure 5C and D). HPLC results also showed that significantly higher levels of soluble oligomers were present in Aβ samples coincubated with curcumin compared to those without curcumin (Figures 5C, D and 6B). At peak levels, soluble Aβ aggregates in curcumin containing samples were over 3 times higher than those present in samples without curcumin (Figure 6B). This oligomer enrichment effect has been previously documented for other polyphenolic compounds[30,37,57,66] and could be due to the stabilization of Aβ aggregation intermediates by curcumin, higher rates of Aβ

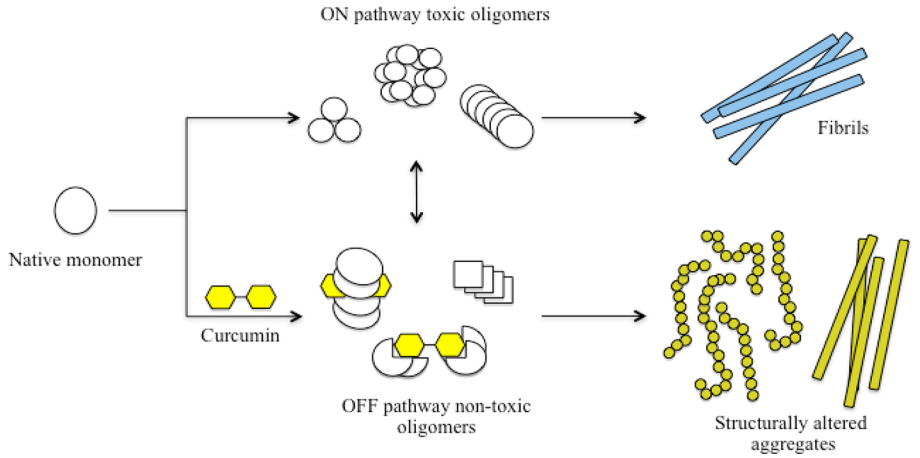

Figure 9. Schematic showing the favorable role curcumin plays in modulating Aβ aggregation by promoting the formation of nontoxic, off-pathway assemblies.[8,9]

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oligomer formation, and/or slower rate of oligomer conversion into mature fibrils.

Although the Aβ soluble oligomers and prefibrillar aggregates formed in the absence and presence of curcumin were morphologically similar, these two populations of aggregates drastically differ in their functional properties. Whereas oligomers and prefibrillar aggregates formed from Aβ incubated alone were toxic, A11 antibody positive, and seeded the formation of straight mature fibrils, the curcumin accumulated oligomers were far less toxic (Figure 7A, unfilled bar), did not show reactivity toward the A11 antibody (Figure 7B), and seeded the formation of distinctly different, wormlike, fibrils (Figure 8B).

Cultured neuroblastoma SH-SY5Y cells exposed to oligomers/prefibrillar aggregates isolated from Aβ coincubated with curcumin showed 90% viability (Figure 7A, unfilled bar), as compared to 50% viability when they were exposed oligomers/prefibrillar aggregates isolated from Aβ incubated alone (Figure 7A, filled bar). Thus, the curcumin-accumulated oligomers were significantly less toxic. These oligomers also did not show reactivity toward the A11 antibody (Figure 7B), which selectively recognizes toxic oligomers and protofibrils of a wide variety of amyloidogenic proteins.[54,63,64] As such, the lack of A11 reactivity indicates that the curcumin accumulated oligomers lack the common structural epitope shared by toxic oligomers of amyloid proteins. This common epitope appears to be a structural feature of the oligomer polypeptide backbone that is independent of amino acid side chains.[65] Moreover, antibody binding effectively abolished oligomer toxicity,[65] suggesting that the structural epitope is responsible for oligomer toxicity and that a common mechanism, such as cell membrane destabilization, might account for oligomer toxicity.[60,65,67] Consistent with these previous findings, our results show that the A11 negative, curcumin-accumulated oligomers were also nontoxic. This lack of A11 reactivity could be due to curcumin binding to Aβ oligomers that blocked the structural epitope on the oligomer for both antibody binding and toxicity. Alternatively, oligomers formed in the presence of curcumin could be sufficiently structurally altered compared to toxic Aβ oligomers such that antibody binding and toxicity were both abolished. A combination of these two effects could also be at play. Although the structural details of the curcumin accumulated oligomer remains unclear, recent computational studies have shed some light to the binding between curcumin and amyloid protein aggregates.[68−70] Molecular simulation of the binding between curcumin and fragments of the Aβ peptide and tau protein showed curcumin interacts with amyloid proteins through a combination of hydrogen-bonding, van der Waals interactions,[68] and π−π stacking with aromatic residue side chains.[69,71] These interactions, on the order of −3 to −10 kcal/mol,[70] stabilizes the curcumin-aggregate complex conformation, and when curcumin was added to preformed aggregates, the compound exerts a general destabilizing effect of the aggregate structure, loosening the packing of β-sheets and enhancing structural fluctuations.[68−70] In our study, the binding of curcumin and the concomitant changes induced to the packing and spacing of secondary structures such as β-sheets in the oligomer could have effectively abolished antibody reactivity and toxicity. Aβ oligomers of similar size and morphology but dissimilar toxicity have been previously reported by Ladiwala and co-workers.[72] In their study, different types oligomers were formed under different peptide concentration and agitation conditions. The less toxic oligomer was found to be devoid of β-sheet structure, insoluble, nonimmunoreactive with oligomer- and fibril-specific antibodies, and incapable of disrupting lipid bilayers.[72]

In this study, differences between the two populations of oligomers were further manifested by the drastically different fibrils the oligomers seeded. Both oligomers rapidly seeded the formation of ThT-positive fibrillar aggregates with similar rates of growth (Figure 8B). However, whereas fibrils seeded from oligomers isolated from Aβ incubated alone showed the characteristic morphological features of mature Aβ fibrils, long, straight, and with periodic twists, fibrils that were seeded by oligomers isolated from Aβ coincubated with curcumin were thin, curly, and short (Figure 8B). This type of curvilinear, wormlike fibrils have been previously observed, for example, apolipoprotein C-II,[73] the N47A mutant of the α-spectrin SH3 (Spc-SH3) domain,[74] and the mouse prion protein.[75] In each case, the amyloid protein also generated straight fibrils under a different set of aggregation conditions than those that generated the wormlike fibrils.[73,74] Although the exact structural basis for the formation of straight vs curly fibrils in each case remains unclear, this phenomenon of amyloid polymorphism has been well-documented.[76,77] Different fibril morphologies have been shown to have different underlying molecular structures and that both morphology and molecular structure are self-propagating when fibrils are grown from preformed seeds.[76,77] In this study, the different fibrils (Figure 8B) similarly reflect differences in the structures of their seeds, oligomers formed in the presence or absence of curcumin. This underlying structural difference between the two oligomers can also lead to their differences in toxicity and antibody reactivity. It is also possible that as curcumin selectively interacts with Aβ oligomers, but not monomers, the presence of bound or complexed curcumin affects the interaction and incorporation of Aβ monomers during fibril growth, leading to distinct fibril morphology. Taken together, our results show that the presence of curcumin did not inhibit Aβ fibril formation, but rather modified the "on-pathway" toxic aggregation process toward an alternative, "off-pathway", mechanism that produced nontoxic aggregates (Figure 9).

Curcumin Exerts Nonspecific Neuroprotective Effect against Aβ Aggregate Induced Toxicity. Another significant finding of this study is that curcumin exerts a nonspecific neuroprotective effect against Aβ aggregate induced toxicity. Consistent with published studies, results from our MTT assay of SH-SY5Y cells showed that Aβ oligomers formed during the first few days of incubation (days 1−4) were particularly toxic (Figure 3, unfilled circles). Cell viability decreased from about 70%, when the cells were exposed to predominantly monomeric 10 μM Aβ (0 day incubation), to 45% when the cells were exposed to 4 day incubated Aβ (Figure 3) that contained approximately 2 mass% soluble oligomers (Figure 6B). This significant decrease in cell viability, by 25%, induced by the low level of soluble Aβ oligomers, ca. 200 nM (monomer basis), points to the potent toxicity of these early aggregates. After 4 days of incubation, although the level of soluble aggregates continued to increase until day 7 to about 5 mass% (Figure 6B), cell viability increased and reached about 60% when the cells were exposed to a 10-day incubated sample containing 60 mass% fibrillar Aβ (Figure 3). Toxicity of oligomeric/prefibrillar Aβ conformers was further confirmed by first isolating these species and then assessing their toxicity. Oligomers and prefibrillar Aβ conformers isolated from a 5-day incubated Aβ sample induced a similar level of toxicity (Figure

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7A, filled bar) compared to an unfractionated Aβ sample (Figure 3).

Although the mechanism of Aβ aggregate induced toxicity remains to be elucidated, it has been hypothesized that Aβ exerts toxicity through a membrane-mediated pathway,[27,60] similar to that proposed for antimicrobial peptides.[78] We have previously shown that the binding of Aβ to membranes compromises membrane integrity,[59−61] which could disrupt ion homeostasis, leading to cell death. To assess cell death due to membrane permeabilization, we carried out live/dead staining assay of SH-SY5Y cells exposed to Aβ aggregates. This assay directly probes cell membrane integrity as the "dead" cells that fluoresce red results from the complexation of a membrane impermeable dye with nucleic acids. Our results show that exposure of the SH-SY5Y cells to a 5-day incubated Aβ sample indeed caused a large increase of membrane compromised cells (Figure 4).

Significantly, pretreating the cells with curcumin reduced both toxicity and cell membrane permeabilization induced by Aβ oligomers. Viability levels of cells that were first pretreated with curcumin (Figure 3, unfilled triangles) were higher for all incubated Aβ samples (0 to 10 days incubated) (Figure 3, unfilled circles), indicative of a nonspecific neuroprotective effect exerted by curcumin against the range of Aβ conformers tested, including monomeric, oligomeric, prefibrillar and fibrillar Aβ. However, curcumin was not able to completely block Aβ toxicity as viability levels were raised to approximately 80% in all cases. The neuroprotective effect of curcumin against toxic Aβ oligomers was specifically demonstrated when pretreated cells were exposed to oligomers fractionated from 5-day incubated Aβ. Viability level of cells was significantly higher for cells that were pretreated with curcumin prior to exposure to fractionated toxic Aβ oligomers (Figure 4D). Pretreating cells with curcumin also reduced the number of red fluorescing, or membrane compromised, cells exposed to Aβ to near the control level, where the cells were not treated (Figure 4A−C). Although the live/dead staining assay does not definitively establish membrane permeabilization as a cause of Aβ induced cell death, it does show that the loss of membrane integrity accompanies cell death. Many studies have supported a membrane mediated, nonspecific, toxicity pathway of amyloid aggregates.[27,28] Our observation of the nonspecific neuroprotective effect of curcumin from both toxicity and live/dead imaging assays is consistent with a membrane-mediated neuroprotective mechanism for curcumin. Although the effect of curcumin on Aβ aggregate-membrane interactions was not precisely determined in this study, we have previously shown that curcumin reduces monomeric Aβ-membrane interactions and subsequent membrane disruptions.[61] Additionally, a number of polyphenols have been shown to protect model lipid membranes from amyloid-aggregate induced disruption.[67,79,80]

CONCLUSIONS

Curcumin has been reported to offer neuroprotection by inhibiting the formation of Aβ oligomers and fibrils and breaking up the preformed fibrils,[50,53] but has also been shown to promote fibril formation.[36,48,54] We have shown that the presence of curcumin did not slow the rate or extent of Aβ40 aggregation into β-sheet enriched fibrillar aggregates. Rather, curcumin promoted the formation of "off-pathway" soluble oligomers and prefibrillar aggregates that were nontoxic. These "off-pathway" nontoxic aggregates are fundamentally altered compared to toxic Aβ oligomers formed from incubating Aβ alone and seeded the formation of drastically different, curly, fibrils. Curcumin also exerted a nonspecific neuroprotective effect, reducing toxicities induced by the range of Aβ conformers tested, including monomeric, oligomeric, prefibrillar, and fibrillar Aβ. The neuroprotective effect is possibly membrane-mediated, as curcumin reduced the extent of cell membrane permeabilization induced by Aβ aggregates. Taken together, our study shows that curcumin exerts its neuroprotective effect against Aβ induced toxicity through at least two concerted pathways, modifying the Aβ aggregation pathway toward the formation of nontoxic aggregates and ameliorating Aβ-induced toxicity possibly through a membrane-protective pathway.

METHODS

Materials. Curcumin, thioflavin-T (ThT), SH-SY5Y cells, Dulbecco's modified Eagle's medium and Ham's F12 (1:1), fetal bovine serum (FBS), dimethylformamide (DMF), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Trifluoroacetic acid (TFA) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ). Phosphate-buffered saline (PBS), Dulbeccos's-PBS (DPBS), and the anti-Aβ-oligomer A11 antibody were purchased from Life Technologies. Other primary antibodies, anti-Aβ(1−16) 6E10, and anti-amyloid fibrils OC were purchased from Abcam (Cambridge, MA) and EMD Millipore (Billerica, MA), respectively. The horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson Laboratory. All other chemicals were acquired from Sigma (St. Louis, MO). All water used was purified with a Synergy UV Millipore purification system (EMD Millipore, Billerica, MA).

Preparation of Aβ and Curcumin Solutions. Aβ40 was synthesized in Dr. Stephen Meredith's lab at the University of Chicago[81] and purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a preparative Zorbax C18 column (Agilent Technologies, CA) at 60 °C with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile) using a (30−60)% linear gradient of B over 50 min.[61] Purified Aβ was then lyophilized (FreeZone 4.5 L Benchtop Freeze-Dry System, Labconco) and stored at −80 °C. The molecular weight of the purified peptide was determined by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) to be 4329.82 Da (expected molecular weight is 4329.86 Da). Purified Aβ was solubilized in DMSO at 10 mg/mL and aliquots were stored at −80 °C and used within 3 months. This stock Aβ solution was subsequently diluted with water or PBS for incubation or toxicity assays. Curcumin was dissolved in 100% DMSO at 5 mM concentration and diluted in water or PBS for incubation or toxicity assays.

Incubation Assays. Stock Aβ solution in DMSO was first briefly sonicated and centrifuged at 15 000*g* for 15 min to remove any large insoluble species. The supernatant containing predominantly monomeric Aβ was then removed and diluted in water or PBS to 100 μM Aβ. Aβ samples (30−100 μL) were then incubated in the absence or presence of 50 μM curcumin (final concentration). In addition to these "unseeded" Aβ samples, seeded Aβ samples were also prepared and incubated. Monomeric Aβ (100 μM) was seeded either with Aβ incubated alone for 5 days or with soluble aggregates fractionated (see below) from Aβ incubated with curcumin for 5 days. The concentrations of Aβ seeds in the solutions were approximately 0.5 or 0.65 μM for Aβ incubated in the absence or presence of curcumin, respectively. All Aβ samples, seeded or unseeded, were incubated quiescently at 37 °C for 0−12 days, with samples taken daily for further analysis as described below, including size exclusion HPLC (SE-HPLC) analysis and fractionation, thioflavin-T fluorescence, transmission electron microscopy (TEM) imaging, circular dichromism (CD) spectroscopy, and toxicity and dot blot assays.

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Size-Exclusion High Performance Liquid Chromatography (SE-HPLC). To analyze and fractionate different size Aβ species in the incubated samples, 100 μL aliquots of incubated Aβ samples were first centrifuged at 15 000*g* for 15 min to remove large insoluble aggregates. The supernatant (65 μL) was then removed and injected onto a BioSep SEC-S3000 column (Phenomenex) using a PBS mobile phase at a flow rate of 0.5 mL/min[61] on an Agilent 1100 HPLC system equipped with a UV/vis detector. Absorbance values at 215 nm were recorded. Under these conditions, the monomeric protein eluted at a retention time of 20.9 min and soluble aggregates that are larger than 700 kDa, the upper separation limit of the SEC column, eluted in the void volume at around 11.5 min. For quantifying the amount of protein in each peak, background subtracted "area under the peak" was obtained from the Agilent ChemStation software. Area under the peak of the monomeric Aβ at day 0 was taken as total amount, or 100% of protein and the amount of insoluble protein was calculated by subtracting total amount of protein by soluble protein peaks.

For seeding (see above) and cell culture experiments (see below), Aβ oligomers eluting in the void volume fraction was collected and concentrated to ∼100 μL using a 10 kDa cutoff membrane filters (Millipore). During concentration, 10−15 mL of PBS was intermittently passed through the membrane. The amount of oligomers in the concentrated sample was either calculated by subtracting the total amount of protein loaded by percentages of insoluble protein and monomers or directly determined by measuring Aβ concentration using a NanoDrop2000 (Thermo Scientific).

Thioflavin-T (ThT) Fluorescence Assay. To detect the formation of Aβ fibrils in unseeded and seeded solutions, 30 μL of Aβ incubated alone or in the presence of 50 μM curcumin was mixed with 270 μL of 50 mM glycine buffer (pH 6.5) containing 5 μM ThT. Fluorescence intensities of the solutions were subsequently measured at excitation and emission wavelengths of 445 and 490 nm, respectively, using a PTI Quantamaster QM-40 spectrofluorometer (Photon Technology Inc.).[57,58] Fluorescence readings were background subtracted by that of ThT alone.

For the fibril disruption assay, curcumin (50 μM final concentration) was added to preformed Aβ fibrils (100 μM Aβ incubated alone for 10 days) and mixed thoroughly. Then, 270 μL of 5 μM ThT was added and fluorescence of the solution was measured immediately.

Transmission Electron Microscopy (TEM). To visualize the morphologies of incubated Aβ samples, TEM images of Aβ adsorbed on glow discharged (Harrick Plasma Cleaner) 6−8 nm carbon films on 400-mesh copper grids (Ted Pella, CA) were obtained. Aliquots of incubated Aβ were first diluted up to 20 times, and 5 μL of diluted samples were put onto copper grids and allowed to adsorb for 2 min. Excess sample solution was removed with blotting paper. The adsorbed samples were then negatively stained with 2% uranyl acetate (Electron Microscopy Sciences) for 2 min after which the staining solution was removed and sample was air-dried (no washing). The samples were then imaged by using a TEM instrument (H-7500, Hitachi, Japan), equipped with an AMT XR60 camera (AMT Imagining), at an accelerating voltage of 80 kV at 40 000-fold magnification in high contrast mode.

Cell Culture and Toxicity Assays. To determine the toxicities of incubated Aβ samples and the effects of curcumin on Aβ-induced neurotoxicity, the MTT reduction assay was used to determine viability of cultured neuronal cells exposed to Aβ samples. Human neuroblastoma SH-SY5Y cells were cultured in DMEM and Ham's F12 supplemented with 10% (v/v) FBS and 1% antibiotics (penicillin and streptomycin) at 37 °C in a 5% CO_2 incubator. Cells were seeded at a density of 20,000 cells/well in 96-well plates (Nunc, Denmark), grown to near 80% confluence, and serum-deprived overnight prior to treatment with incubated Aβ samples. An individual aliquot of 35 μL of 100 μM Aβ incubated either alone or in the presence of 50 μM curcumin at various times (0 to 10 days) were diluted 10-fold in culture medium and added to cells for 16 h. In other words, the cells were treated with incubated solutions of 10 μM Aβ alone or 10 μM Aβ with 5 μM curcumin. In addition to treating cells with incubated Aβ samples that contain a mixture of different aggregate states (monomeric, oliogmeric, and fibrillar Aβ), cells were also treated with SE-HPLC fractionated soluble oligomers or a mixture of oligomers and monomers from Aβ alone or Aβ coincubated with curcumin solutions. For curcumin pretreatment toxicity assays, 12−16 h serum-deprived cells were pretreated with 5 μM curcumin for 3 h, rinsed once with culture media, and exposed to 10 μM Aβ incubated alone for 0−10 days. Appropriate controls of curcumin (5 μM) and DMSO (∼0.5%) were also included during treatment. For the MTT reduction analysis, a 20 μL aliquot of 5 mg/mL MTT solution in PBS was first added to each well containing cells exposed to Aβ samples and incubated. After 2 h, 100 μL of solubilization buffer (20% SDS solution in 50% (v/v) DMF at pH 4.7) was added. The absorbance at 570 nm was recorded after 4−6 h using a microplate reader (Spectra Max 190, Molecular Devices).

Separately, cell membrane integrity based live/dead assay was also performed. In this experiment, cells were cultured in 6-well plates to a density of 150 000 cells/well and treated with 1 mL of Aβ samples for 16 h. The cells were then rinsed twice with D-PBS and incubated with a mixture of 0.5 μM calcein-AM and 2.5 μM ethidium homodimer-1 in D-PBS for 30 min. After incubation, fluorescent and phase images were taken using an inverted microscope (Nikon Ti Eclipse, Japan) equipped with a 40× objective lens and appropriate filters (FITC for calcein-AM and Texas Red for ethidium homodimer-1). The number of dead cells was counted from five to six random fluorescence images.

Dot Blot Assay. Dot blot assay was carried out using three different antibodies that are either Aβ sequence specific (anti-Aβ 6E10), oligomer specific (antioligomer A11), or fibril specific (antiamyloid fibrils OC). Monomers (freshly dissolved Aβ sample), toxic Aβ oligomers (oligomers formed from incubating 100 μM Aβ alone for 5 days), curcumin accumulated Aβ oligomers (oligomers formed from coincubating Aβ with 5 μM curcumin for 5 days and then fractionated with SE-HPLC), and Aβ fibrils (insoluble aggregates removed from Aβ incubated alone for 10 days) were blotted onto polyvinylidene difluoride membrane (Bio-Rad Laboratories, CA) and air-dried. After 1 h blocking in 5% milk containing tris-buffered saline (TBS) with 0.2% (v/v) tween (TBS-T) at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies (6E10, 1:5000 dilution; A11, 1:1000 dilution; and OC, 1:1000 dilution). The membranes were then washed three times with TBS-T and incubated with HRP-conjugated secondary anti-rabbit or anti-mouse antibodies for 1 h at room temperature. After five washes, the antigen and antibody binding was detected using the SuperSignal West Dura Extended Duration chemiluminescent substrate (Pierce, IL).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00214.

Far-UV circular dichroism spectra of monomeric and fibrillar Aβ in the presence and absence of curcumin (PDF)

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Author Contributions

E.Y.C. and A.T. designed the project. A.T. performed the experiments. S.D.J. carried out the T.E.M. imaging and analysis. All authors contributed to the preparation of the manuscript and approve the final version.

Author Contributions

All authors contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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■ ABBREVATIONS

Aβ, amyloid-β peptide; AD, Alzheimer's disease; CD, circular dichroism spectroscopy; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; RP-HPLC, reverse-phase high performance liquid chromatography; SE-HPLC, size exclusion-HPLC; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TEM, transmission electron microscopy; TFA, trifluoroacetic acid

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