ABSTRACT There are three specific regions in the Amyloid beta (Aβ) peptide sequence where variations cause enhanced toxicity in Alzheimer’s disease: the N-terminus, the central salt bridge, and the C-terminus. Here, we investigate if there is a close conformational connection between these three regions, which may suggest a concerted mechanism of toxicity. We measure the effects of Zn$^{2+}$ and curcumin on Aβ$_{40}$, and compare these with their previously reported effects on Aβ$_{42}$. Aβ$_{42}$ and Aβ$_{40}$ differ only near the C-terminus, where curcumin interacts, while Zn$^{2+}$ interacts near the N-terminus. Therefore, this comparison should help us differentiate the effect of modulating the C- and the N-termini. We find that curcumin allows fibril-like structures containing the salt bridge to emerge in the mature Aβ$_{40}$ aggregates, but not in Aβ$_{42}$. In contrast, we find no difference in the effects of Zn$^{2+}$ on Aβ$_{40}$ and Aβ$_{42}$. In the presence of Zn$^{2+}$, both of these fail to form proper fibrils, and the salt bridge remains disrupted. These results indicate that modulations of the Aβ termini can determine the fate of a salt bridge far away in the sequence, and this has significant consequences for Aβ toxicity. We also infer that small molecules can alter oligomer-induced toxicity by modulating the aggregation pathway, without substantially changing the final product of aggregation.

INTRODUCTION

Toxicity associated with amyloid beta (Aβ) aggregation is linked to Alzheimer’s disease (1–4), but the mechanism of toxicity has remained unknown. Nature provides indications that Aβ toxicity has a structural origin. For example, Aβ$_{40}$ and Aβ$_{42}$ are the two major isoforms of Aβ found in the brain plaques of Alzheimer’s patients. They are identical except for the two extra amino acids at the C-terminus of Aβ$_{42}$, which make it fold differently (5–9), and also make it more aggregation-prone and toxic (10). At least two other regions also seem to be important for determining toxicity. Mutations in Aβ that lead to early onset AD almost exclusively map to one of these two regions: the N-terminus (first seven residues, which are loosely structured at best); or the central region (residues 21–23) (11–13), which in wild-type Aβ$_{40}$ contains a salt bridge. It is interesting to hypothesize that these three regions constitute conformationally connected arms of the same toxic mechanism.

Curcumin, a biphenolic compound known to interact with Aβ and alter its properties (14–18), provides a natural handle to test this hypothesis. Curcumin is known to modulate Aβ toxicity (17,19,20), and its most prominent site of interaction is the C-terminal region, though it also interacts with some residues at the N-terminal region (as shown for Aβ$_{42}$ (21,22)). Zn$^{2+}$, another well-known modulator of Aβ aggregation and toxicity (23,24), provides an ideal comparison. It is thought to interact almost exclusively with the Histidine residues near the N-terminal (25–27). It therefore should have very similar effects on Aβ$_{42}$ and Aβ$_{40}$. Both Zn$^{2+}$ and curcumin are known to interact with the toxic oligomers of Aβ$_{42}$, and sequester them by accelerating their precipitation, which yield similar nonfibrillar aggregates. Significantly, the salt bridge between Lys$^{28}$ and Asp$^{23}$, a key structural feature of mature C-terminal-amidated Aβ fibrils (28), is lacking in these Aβ$_{42}$ aggregates (21,29). It was inferred that these agents disrupt the salt bridge. Notably, a large fraction of the mutations associated with early onset AD occurs in the two neighboring residues...
(E22 and D23) in the salt-bridge region (13). We note that recent works on two different preparations of the Aβ42 fibril have suggested a different salt bridge between K28 and the terminal carboxylate (9,30). However, in alternative preparations, such as those where the C-terminal is amidated, lys28 does form a salt bridge with D23 (29). In addition, we have also shown that there are significant structural differences between the transient toxic oligomers and the less toxic mature fibrils of Aβ40 in the salt-bridge region (1). Hence, the perturbation of the salt bridge may be directly linked to the alteration of toxicity.

The above-mentioned reports suggested that the terminal regions of Aβ42 can control the fate of the central salt bridge, but they could not rule out direct involvement of Zn2+ or curcumin with the central region. However, if the effect of curcumin on the salt bridge is different for Aβ40 and Aβ42, then it will strongly indicate that the state of the residues at the C-terminus has a strong role to play in determining the state of the salt bridge. Here, we follow the fate of the salt bridge in separate preparations of Aβ40 incubated with curcumin and Zn2+, respectively, using solid-state nuclear magnetic resonance (ssNMR) tools. In addition, we follow the size of the soluble aggregates (with fluorescence correlation spectroscopy, FCS), the mesoscale morphology of the aggregates (with transmission electron microscopy, TEM), and the toxicity of Aβ40. We then compare these results with those reported for Aβ42. We draw three important inferences from our results: first, the interaction of curcumin with the C-terminal of the peptide can determine the fate of the salt bridge far away in the sequence; second, the fate of the salt bridge is associated with the toxicity of the peptide, and the mesoscale morphology of the aggregates; and third, a small molecule such as curcumin can act like a catalyst in the Aβ40 aggregation pathway, changing only the nature of the intermediate, but leaving the end-state nearly unaffected.

**MATERIALS AND METHODS**

**Materials**

Rink amide MBHA resin LL, Fmoc (N-(9-fluorenyl) methoxycarbonyl) protected amino acids, O-benzotriazolyl-N,N,N,N-tetramethylenuroniumhexafluoro phosphate, and trisopropylsilane were purchased from Advanced ChemTech (Louisville, KY). 1-Hydroxybenzotriazole, N,N-dimethylformamide, collidine, TFA, tert-butyl methyl ether, acetonitrile, and isopropyl alcohol were obtained from S.D. Fine Chemicals (Mumbai, India). 1,8-Diazabicyclo[5.4.0]undec-7-ene, N-methyl morpholine, piperdine, hexafluoropropionic acid, thio-T, DMSO, 5 (6)-carboxytetramethylrhodamine-N-succinimidyl ester, and the buffer salts are purchased from Sigma-Aldrich (St. Louis, MO). Phenol, N,N-diisopropylcarbodiimide, ethane dithiol, thioanisole, and trifluoroethanol are purchased from Fluka (Sigma-Aldrich). The isotopically labeled amino acids were purchased from Euriso-top (St. Aubin, France). All animal experimental protocols were approved by the Institute Animal Ethics committee.

**Aβ40 sample preparation**

Wild-type Aβ40 and three different schemes of 15N-, 13C-labeled Aβ40 were synthesized in a solid phase peptide synthesizer and further purified by a reverse-phase HPLC. The scheme 1 (S1) Aβ40 had Glu11, Phe19, Ala30, Leu34, Val36, and Gly38 isotopically labeled with 15N, 13C; scheme 2 (S2) had Ala2, Val12, Phe20, Asp23, Ser26, Lys28, and Met35 as 15N, 13C labeled; and scheme (S3) had Asp23 and Lys28 labeled with 15N, 13C. Purified Aβ peptides were initially dissolved in pH 11 water (adjusted by NaOH) to prepare 2 mM stock solutions. To grow Aβ40 fibrils at pH 7.4, the stock solutions were diluted five times in HEPES buffer (20 mM HEPES, 146 mM sodium chloride, 5.4 mM potassium chloride, 1.8 mM CaCl2, 2H2O, 0.8 mM MgSO4·7H2O) maintained at pH 7.4 to give final peptide concentrations of 400 μM at pH 7.4. To grow Aβ40 fibrils in the presence of Zn2+ ions, the 2 mM stock solutions of Aβ40 were diluted five times in HEPES buffer (pH 7.4) containing 500 μM ZnCl2, giving a final solution that had 400 μM of both ZnCl2 and the respective peptides. For aggregates of Aβ40 in the presence of curcumin, a 4 mM stock solution of curcumin was also prepared by dissolving it in pH 11.0 water. A quantity of 0.1% of uranyl acetate was added for 5 min for sample staining. A final solution of 2 mM stock solutions of Aβ40-Cur (either 400 μM Aβ40, or 8 μM of curcumin) were aggregated for four days in HEPES buffer at pH 7.4 such that total volume was 7.5 mL. This final solution containing 400 μM Aβ40, 80 μM curcumin, and 100 nM RAJα was incubated at room temperature (25°C) for four days with mild rotation (10 rpm). For the early aggregates of Aβ40 in the presence of curcumin, we prepared the sample in a similar way to that described above, but with centrifugation (2700 × g) after 30 min of pH drop (from pH 11 to 7.4). The final pH of the solution was 7.4. The precipitate was lyophilized before packing into the NMR rotor. To grow Aβ-Cur aggregates at lower concentrations (40 μM of Aβ and 8 μM of curcumin), freshly prepared stock solutions of Aβ and curcumin (as described above) were diluted 10 times at pH 11, premixed with 7.5 μL RAJα (100 μM) for 5 min at pH 11, followed by dilution with HEPES buffer (up to 7.5 mL, pH 7.4), and incubation at room temperature (25°C) for four days with mild rotation (10 rpm).

**Fluorescence correlation spectroscopy**

Size of the Aβ species in solution was determined by FCS measurements following procedures similar to Sengupta et al. (31). The measurements were performed with an instrument constructed in-house (31). FCS data were fitted with a discrete component diffusion model using Origin 7.5 software (OriginLab, Northampton, MA). The diffusion times were converted into hydrodynamic radii (Rg) using rhodamine B (assumed Rg = 0.57 nm) as a calibrant. We used the MEMFCS (32) fitting routine developed specifically for such measurements to obtain a size distribution from the FCS data in a model-free manner.

**TEM**

Aβ40 (400 μM) and Aβ40-Cur (either 400 μM Aβ40 + 80 μM curcumin or 40 μM Aβ40 + 8 μM curcumin) were aggregated for four days in HEPES buffer. Ten milliliters of these solutions were added to carbon-coated, 100-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). The extra water was blotted with tissue paper after 2–3 min, and a few mild washings were done with Milli-Q water (Millipore, Billerica, MA). A quantity of 0.1% of uranyl acetate was added for 5 min for sample staining. The grids were dried under an infrared lamp and examined in the LIBRA 120 EFTEM (Carl Zeiss, Oberkochen, Germany).

**Solid-state NMR**

The ssNMR measurements were performed on amyloid aggregates of Aβ40 peptides (400 μM) containing isotopically labeled amino acids at specific positions grown in the presence and absence of Zn2+ and curcumin. The aggregates were pelleted by ultracentrifugation (2700 × g at 20°C), washed with deionized water twice, rapidly frozen using liquid nitrogen,
and lyophilized. Powders containing Aβ(40) peptides were packed as such without any rehydration into either 2.5, 4, or 3.2 mm magic-angle-spinning (MAS) rotors. All the ssNMR measurements were performed in a 700 MHz model No. AVIII NMR spectrometer (Bruker, Billerica, MA) using 2.5, 3.2, or 4 mm triple-resonance MAS probes. Cross polarization from 1H to 13C was implemented using a linear ramped radio-frequency field (33) centered ~60–85 kHz on the 1H channel and ~35–55 kHz on the 13C channel depending upon the MAS frequency, νr (different for different experiments, as stated). Cross-polarization contact pulse time was kept between 1.5 and 4.0 ms. A swept-frequency, two-pulse phase modulation (φ = 10°, 15°) decoupling scheme (34) with an 1H radio-frequency field strength between 85 and 95 kHz was used to accomplish efficient 1H-13C dipolar decoupling. Two-dimensional (2D) 13C-13C through-space NMR spectra were recorded using a second-order dipolar recoupling, phase-alternated recoupling irradiation scheme (PARIS-xy) (35) with an 1H radio-frequency field strength between 85 and 15 kHz were used for S1 Aβ. For the S2 peptide, 2D 13C-13C through-space correlation NMR spectra were recorded with a PARIS-xy (m = 1, N = 0.5) recoupling scheme using mixing periods of 40 and 400 ms at a νr of 15 kHz.

NMR data analysis
All one-dimensional (1D) spectra were processed and analyzed using the software TopSpin 2.0/3.1 (Bruker). All 2D spectra were processed with TopSpin 2.0/3.1 and analyzed using the software CCP NMR Analysis 2.2.2 (http://www.ccpn.ac.uk/homepage). The data were zero-filled in the t1 and t2 dimensions to 512 and 4096 points, respectively. A mixed sine/cosine (φ = π/3 at t = 0) apodization function was used in each dimension. All the spectra were externally referenced to tetramethylsilane in methanol (36).

To calculate average chemical-shift change (Δδ) values, 13C chemical-shifts of isotopically labeled amino acids in Aβ(40) fibrils were first subtracted from that of Aβ(40)-Cur aggregates. These chemical-shift differences for α, β, and carbonyl carbons (the backbone), and the remaining carbons (the side chain), were then averaged over the number of carbons constituting the backbone and side chain, respectively, to obtain average chemical-shift change (Δδ).

Cell viability assay
The cell toxicity assay was performed on 3-day-old rat primary cortical neuronal cultures grown in 96 well plates. All the animal procedures were approved by the TIFR Animal Ethics Committee. On day 4, the primary neurons were incubated with cell culture media containing Aβ solutions (4–6 wells each at two different concentrations—first set with 400 μM Aβ(40); 400 μM Aβ(40) + 30 μM curumin; 80 μM curumin; and second set with 40 μM Aβ(40), 40 μM Aβ(40) + 8 μM curumin, 8 μM curumin) for 48 h. For the Zn2+ case, the cells were incubated in culture media containing Aβ solutions (100 μM Aβ(40), 100 μM Aβ(40) + 8 μM Zn2+, 100 μM Aβ(40), 100 μM Aβ(40) + 8 μM Zn2+ for 48 h. At the end of 48 h, cell media was removed and the cells were washed with Thomson’s buffer at pH 7.4 (20 mM HEPES, 146 mM NaCl, 5.4 mM KCl, 2.3 mM CaCl2, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, 5 mM Glucose). Cells were then incubated with Thomson’s buffer (pH 7.4) containing 0.01 mg/mL propidium iodide (PI) for 10 min. PI was removed and cells were washed three times with Thomson’s buffer and subsequently fixed with 4% PFA solution. The cells were then imaged for PI under an epifluorescence microscope (Axiovert 200; Carl Zeiss) using a 40× objective. The images were captured with a charge-coupled device camera (AxioCam; Carl Zeiss). Image analysis was performed for several fields in multiple dishes (at least 12 for each peptide) from three independent repeats and scored for the total number of cells (transmission images) and the number of dead cells (PI fluorescent spots). The ratio of cells that were alive (PI negative) to total cells was reported as viability. The cell viability expressed as percentage was normalized with respect to control cell viability, where the control cell viability was assumed to be 100% (actual viability in control cells was 78 ± 3%). Data are expressed as mean ± SE. Student’s t-test was performed to test the significance of the difference of the mean number of deaths from different samples.

RESULTS
We first report the studies highlighting the effect of curcumin and Zn2+ on the nonstructural aspects, i.e., toxicity, aggregation kinetics, and the nature of the soluble aggregates. Structural details obtained from NMR studies are reported later.

Aβ toxicity
The effect of curcumin on Aβ(40)-induced toxicity on rat primary cortical neuronal cultures was probed according to the protocol described in the Materials and Methods. The results of the cell viability measurement, expressed as a percentage of the control, are shown in Fig. 1 A. Aβ(40) was found to be significantly toxic at a concentration of 400 μM (p < 0.001; Fig. 1 Ab). Presence of 80 μM curumin significantly reduces the toxicity (p < 0.001; Fig. 1 Ac). However, because curcumin itself reduces the viability at such high concentrations (Fig. 1 Ad), the assays were also performed using 40 μM of Aβ(40) and 8 μM of curumin maintaining the ratio at 5:1, and still maintaining the Aβ concentration well above the saturation concentration (16 μM (37)). The toxicity of 40 μM of Aβ(40) is lower but still substantial (p < 0.001; Fig. 1 Ae). A quantity of 8 μM of curumin significantly reduces this toxicity (p < 0.001; Fig. 1 Af), although it is barely toxic by itself (Fig. 1 Ag). Similar effects on toxicity have already been reported by us in the case of Aβ(42), where 80 μM curumin alleviates the toxicity induced by 400 μM of Aβ(42) to a similar extent (21). We note that curcumin dose-response curves have been reported earlier, and concentrations in this range were found to be effective in reducing toxicity (17–19,38,39). The effect of Zn2+ on the toxicity of Aβ(40) and Aβ(42) was measured in a similar way. The concentrations of Aβ and Zn2+ used were 100 and 8 μM, respectively. In the case of Aβ(40) in presence of Zn2+, the viability was ~80%; in the case of Aβ(42) (p < 0.001; Fig. 1 B), it was 50%.

Therefore, toxicity measurements do not show any qualitative difference between the effects of curcumin and Zn2+ on either Aβ(40) or Aβ(42).

Size distribution of the soluble oligomers
FCS was used to measure the size (hydrodynamic radius) distribution of the soluble species. A quantity of 40 μM Aβ containing 100 nM of rhodamine-labeled Aβ(40) (RHB-Aβ40) was coincubated with 8 μM curumin. FCS autocorrelation curves were measured 4 h after aggregation.
was initiated with a pH drop from pH 11 to pH 7.4. The results are shown in Fig. 1, C and D. The FCS data obtained from 40 μM Aβ40 in presence (triangles) and absence (circles) of curcumin (Fig. 1 C) were fitted with a quasi-continuous size distribution model using the MEMFCS fitting routine (32) (Fig. 1 D). Here, the abscissa represents the hydrodynamic radii in nanometer (this axis is calibrated by measuring the diffusion of rhodamine B in water, which has a hydrodynamic radius of 0.57 nm (40)), and the ordinate represents the relative population weighted by the square of the particle brightness (32). The first peak at ~2 nm corresponds to the monomeric and the small oligomeric species, while the second peak at ~200 nm corresponds to the larger, quasi-stable, soluble aggregates. We observe that the large soluble aggregates are no longer detectable when curcumin is present in the solution (Fig. 1 D), implying that curcumin accelerates the precipitation of these aggregates and they grow in the precipitate phase. We also observe a small shift and a narrowing of the first peak, which indicate a simultaneous decrease of the population of smaller oligomers.

We have previously shown that Zn2+ also rapidly precipitates the soluble aggregates of Aβ40 and Aβ42 (23), indicating that both curcumin and Zn2+ have similar effects on both the peptides.

TEM measurements of the fibrils

TEM images of Aβ40 and Aβ42 were recorded at two different peptide/curcumin concentrations: 40 μM (Fig. 2 B for Aβ40; Fig. 2 E for Aβ42) and 400:80 μM (Fig. 2 C for Aβ40; Fig. 2 F for Aβ42). The Aβ40 aggregates grown in the presence of curcumin do form fibril-like structures, in some cases growing beyond 100 nm (marked by solid rectangles in Fig. 2). However, they are much shorter than the fibrils grown without curcumin. In contrast, the aggregates of curcumin-incubated Aβ42 are shorter and more disordered (Fig. 2, E and F), consistent with what we have previously reported (21). A measurement of the length distribution of the aggregates of Aβ40 versus Aβ42 quantitatively shows that the average length of the fibrils is indeed much larger for Aβ40 (Fig. S1). We have earlier shown that Zn2+ completely disrupts fibril formation (29). In summary, while both curcumin and Zn2+ disrupt fibrilization of both the Aβ peptides, curcumin-incubated Aβ40 shows less disruption than others.

Atomic-level contacts probed by solid-state NMR

We then probed the atomic-level structural changes in Aβ40 aggregates induced by Zn2+ and curcumin, respectively, using ssNMR. We designed two different 13C and 15N labeled amino-acid schemes to monitor different regions of the peptide including the turn, the tail, and the two hydrophobic arms. Scheme 1 (S1) covers mainly the hydrophobic arms and has Glu1, Phe19, Ala30, Leu34, Val36, and Gly38 isotopically labeled. Scheme 2 (S2) includes mainly the turn (including the salt-bridge forming residues) and tail regions and has Ala2, Val12, Phe20, Asp23, Ser26, Lys28, and Met35 isotopically labeled. We performed separate ssNMR
measurements on specimens incubated in curcumin for 30 min and four days. We focused on the two distal interresidue contacts established in the mature fibrils of Aβ40, namely, the contact between F19 and L34, and the salt-bridge contact between D23 and K28. It is known that the F19–L34 contact is already established in the early oligomers, but the salt-bridge region appears different in this species (1). Fig. 3 A shows an overlay of 2D 13C-13C PARIS-xy (m = 1, N = 2) spectra of Aβ40 (black online) and Aβ40-Cur (incubated for four days; red online) aggregates of scheme S1 recorded with 400 ms of mixing time. Fig. 3 B shows the same for Aβ40 (scheme S1) incubated with Zn2+. These spectra do not exhibit any drastic changes in peak positions or intensities compared to the spectra obtained from Aβ alone. The presence of the cross peak, indicating the spatial contact between aromatic carbons (δel/εl) of Phe19 and side-chain carbons (γδ) of Leu34 (highlighted with dotted square), suggests that the hairpin shape remains largely unperturbed. However, the cross-peak intensity in the case of Aβ40-Zn2+ aggregates is weaker than that in the Aβ40-Cur aggregates. For Aβ42-Zn2+ and Aβ42-Cur, the cross-peak intensities largely remain similar to the wild-type Aβ42 (21,29). Overall, curcumin and Zn2+ do not strongly perturb the core hydrophobic region of either of the peptide variants.

We then probed the changes induced by Zn2+ on the sample Scheme 2 (S2). Fig. 4 A shows an overlay of 2D 13C-13C PARIS-xy (m = 1, N = 0.5) spectra of Aβ40 (black online) and Aβ40-Zn2+ (red online) aggregates. Even though the two spectra in Fig. 4 A appear similar, significant changes in the nature (pertaining to conformational changes), chemical shift, as well as the intensity of cross peaks corresponding to amino acids Asp23 and Lys28, are observed. Cross peaks belonging to Lys28 are not only shifted, but are also considerably weaker. For the wild-type Aβ40, in the 13C 1D spectrum, Asp23 shows two peaks at 178.1 ppm and at 180.1 ppm. Similarly Nζ of Lys28 shows two peaks at 38.2 and 34 ppm. A frequency-selective REDOR experiment was performed to confirm that the Cγ peak of Asp23 at 180.1 ppm forms a salt bridge with Nζ of Lys28 (Fig. 5 D). Fig. 4 B shows the 1D sum projection extracted from the 2D 13C-13C spectra in the carbonyl region of Aβ40-Zn2+. In the case of fibrils grown in the presence of Zn2+, the peak corresponding to the salt-bridge-forming carbonyl group (~180 ppm) of the side chain of Asp23 is replaced by a broad peak (~178 ppm) (29). Also, the 15N 1D spectra in Fig. 4 C, shows a change in the chemical shift from 38.2 ppm (fibril) to 34 ppm (fibril-Zn2+). This redistribution of Asp23 and Lys28 population for Aβ40-Zn2+ is very similar to what was observed for Aβ42-Zn2+ previously (29). REDOR experiments showed that for Aβ42-Zn2+, the ~178 ppm peak corresponds to a non-salt-bridge-forming species. The broad envelope of Asp23 Cγ peak (~178 ppm) in Aβ40-Zn2+ prevented us from performing REDOR experiments. However, as the chemical shift pattern of Asp23 and Lys28 for Aβ40-Zn2+ is very similar to that of Aβ42-Zn2+, we infer that Aβ40-Zn2+ also does not have the salt bridge. Based on the chemical shift value, the non-salt-bridge-forming Lys28 side-chain Nζ seems to be uncharged (41), which would indicate a local pKa change for the side chain of Lys28. However, additional experiments will be required to determine the charge state of Nζ. Fig. 4, D and E, shows an overlay of Aβ40 (solid), Aβ40-Zn (shaded), and Aβ40 small oligomer spectra (adapted from Sarkar et al. (1); dark dotted shading). This highlights the peaks belonging to the salt-bridge-forming Asp23 side chain. The conformations of Asp23 and Lys28 are very similar in the small oligomers and in the Zn2+ captured Aβ40, suggesting that Zn2+ captures the salt-bridge region in the oligomeric state, and arrests any further development of this region even as aggregation proceeds. Aβ42-Zn2+ aggregates also showed a very similar result (29).
Fig. 5 A shows an overlay of 2D $^{13}$C-$^{13}$C PARIS-xy ($m = 1, N = 0.5$) spectra of $\mathrm{A}\beta_{40}$ (black online) and $\mathrm{A}\beta_{40}$-Cur (red online) aggregates of isotopic labeling scheme 1 recorded with a mixing time of 400 ms. (Dotted rectangles) Cross peaks between aromatic carbons of Phe$^{19}$ and side-chain $\gamma$/$\delta$ carbons of Leu$^{34}$ are shown, and (B) corresponding spectra with Zn$^{2+}$. (C) The cross peaks highlighted in (A) extracted as a sum projection of rows between 20.5 and 27 ppm from the 2D spectrum of $\mathrm{A}\beta_{40}$ (black online) and $\mathrm{A}\beta_{40}$-Cur aggregates (red online) are depicted, with (D) a similar sum projection for Zn$^{2+}$ aggregates. To see this figure in color, go online.

Fig. 5 A shows an overlay of 2D $^{13}$C-$^{13}$C PARIS-xy ($m = 1, N = 0.5$) spectra of $\mathrm{A}\beta_{40}$ (black online) and $\mathrm{A}\beta_{40}$-Cur (red online) aggregates (four days old) recorded from isotopic labeling scheme 2 ($S_2$) with a mixing time of 40 ms. The Asp$^{23}$ and Lys$^{28}$ cross peaks overlap with each other. Fig. 5, B and C, shows overlay of $^{13}$C 1D spectra of the carbonyl region and $^{15}$N 1D spectrum, respectively, of $\mathrm{A}\beta_{40}$ (black online) and $\mathrm{A}\beta_{40}$-Cur (red online) aggregates. The $\delta$-carbon of Asp$^{23}$ shows a major peak at 180.5 ppm and a minor peak at 179 ppm (Fig. 5 B). $\zeta$-Nitrogen of Lys$^{28}$ gives rise to two maxima at 36.2 and 38.3 ppm in $\mathrm{A}\beta_{40}$ aggregates, and similar peaks for $\mathrm{A}\beta_{40}$-Cur aggregates as well (Fig. 5 C). Salt-bridge formation was further probed by fs-REDOR measurements between $\gamma$-carbon of Asp$^{23}$ and $\zeta$-nitrogen of Lys$^{28}$, the results of which are shown in Fig. 5 D. For an isolated $^{13}$C-$^{15}$N pair, the $S_1/S_0$ value is expected to reach one-half of its initial value at $\tau_{\text{mix}} = 0.257 \text{ ms} \times (R_{\text{NC}}/\text{Å})^3$, where $\tau_{\text{mix}}$ is the REDOR dephasing time, and $R_{\text{NC}}$ is the internuclear distance (28,42). REDOR dephasing curves of Asp$^{23}$ in both $\mathrm{A}\beta_{40}$ and $\mathrm{A}\beta_{40}$-Cur aggregates yielded a half-maximum ($S/2S_0$) between 17.1 and 21.2 ms, corresponding to a $^{13}$C-$^{15}$N distance of 4.0–4.4 Å. This shows that Asp$^{23}$ in both cases forms a salt bridge with Lys$^{28}$. The presence of the salt bridge in $\mathrm{A}\beta_{40}$-Cur aggregates distinguishes it from $\mathrm{A}\beta_{42}$-Cur (21), and also from $\mathrm{A}\beta_{42}$-Zn (29) and $\mathrm{A}\beta_{40}$-Zn aggregates.

We then tested if the changes observed at four days are present from the early stages of aggregation. We separately recorded 2D $^{13}$C-$^{13}$C correlation spectra from the early aggregates (30 min) of $\mathrm{A}\beta_{40}$, in the presence of curcumin (Fig. 5 E). We observe that the peak corresponding to the salt-bridge conformation of Asp$^{23}$ (~180.4 ppm) is missing at 30 min. Fig. 5 E shows significant similarity in spectra yielded by these 30-min-old $\mathrm{A}\beta_{40}$-Cur aggregates and the small oligomers of pure $\mathrm{A}\beta_{40}$ (shown in dotted blue online) (1), showing that initial interaction of curcumin with the small oligomers of $\mathrm{A}\beta_{40}$ is similar to that of Zn$^{2+}$.

Fig. 5 F shows changes in chemical shifts in the $\alpha$, $\beta$, and carbonyl carbons of all conformations of labeled amino acids in $\mathrm{A}\beta_{40}$, when aggregated in the presence of Zn$^{2+}$ (Fig. 6 A) and curcumin (Fig. 6 B). The secondary chemical shift data show that in $\mathrm{A}\beta_{40}$-Zn$^{2+}$ aggregates, the $\beta$-sheet extends to 1–2 more residues compared to $\mathrm{A}\beta_{40}$. At the C-terminal, many of the amino acids seem to have
unchanged conformation in Aβ40-Zn²⁺, such as V36, M35, A30, and G38. However, there is more structural heterogeneity in the N-terminal region, as indicated by the multiple peaks observed for A2. E11 and V12, which also show stronger and more heterogeneous cross peaks compared to the Aβ40 fibrils. In Aβ40 fibrils the N-terminal β-sheet region starts from the E11 residue and extends up to D23, whereas, in Aβ40-Zn²⁺, E11 and V12 have one of the conformations in a non-β-sheet state (Fig. S2). A clear Cβ-CO cross peak of F20 in Aβ40-Zn²⁺ (Fig. 4 A) suggests a more rigid conformation in the surrounding region, compared to Aβ40 alone. In all the cases, the main difference arises due to changes in the chemical shift of the carbonyl carbon. Aβ42-Zn²⁺ aggregates did not show any chemical shift change or population redistributions (except for D23, K28, and S26 residues) (29).

Aβ40-Cur aggregates do not show any strong differences at the N-terminal in comparison with Aβ40. There is a small chemical shift difference for A2, and for V12 (Fig. 5 A). In both, the β-sheet starts forming E11 on the N-terminal (Fig. S2). The backbone of E11, V12, and side chain of V12 and F19 undergo structural changes in the presence of curcumin. As observed in the case of Aβ42, curcumin has an effect on the chemical shift of V12 and E11 (21). In the C-terminal, the effect of curcumin on V36 is similar in both Aβ42 (21) and Aβ40, with curcumin causing population redistribution among two conformers of V36 leading to the total disappearance of V36'. However, unlike Aβ42-Cur (21), we did not see curcumin causing any changes in the spectra of L34 and G38 in Aβ40.

Fig. 6. C and D, summarizes the structural elements in Aβ40 that appear to interact with Zn²⁺ and curcumin, respectively. All amino acids labeled with ¹³C and ¹⁵N are marked with double circles. Amino acids that participate in a significant structural change, i.e., those suffering a chemical-shift change of Δδ ≥ 0.5 ppm, are highlighted in filled light blue online. Amino acids showing changes in relative population among different structural conformers are highlighted with a dotted red online. Filled green online Asp²³ and Lys²⁸ that undergo a structural change only in the early oligomers, but not in the mature aggregates. The full table of chemical shifts is provided in Table S1.
DISCUSSION

In this work we investigate the structural correlates of Aβ toxicity reduction caused by Zn$^{2+}$ and curcumin, focusing on the central salt bridge observed in Aβ fibrils. Further, we compare these results for both Aβ$_{40}$ and Aβ$_{42}$, to probe the conformational connection between the C-terminal and the central salt bridge. We first establish the reduction of toxicity of the Aβ species on which we performed the experiments. We then measure the stability of the soluble oligomers, and the morphology of the mature aggregates, and investigate the atomic level changes in the structure of the hydrophobic core and of the salt-bridge regions of the peptide. As we discuss below, these results lead to a consistent picture of the connection among the state of the salt bridge, the fibril morphology, and toxicity. Most importantly, we find that interactions at the C-terminus can alter the state of the salt bridge, located far away in the sequence, and also in physical distance according to most of the structural models (21,29).

Reduction of toxicity is likely due to the precipitation of the oligomeric species

FCS measurements described in Fig. 1 suggest that a major effect of curcumin is to precipitate the soluble aggregates of Aβ. While the large soluble aggregates (~200 nm) are completely precipitated by curcumin, even the smaller aggregates are affected. Similar observations were made by

FIGURE 5 Effect of curcumin on the turn region of Aβ. (A) Shown here is an overlay of 2D $^{13}$C-$^{13}$C PARIS-xy $(m=1, N=0.5)$ spectra of Aβ$_{40}$ (black online) and Aβ$_{40}$-Cur (red online) aggregates of isotopic labeling scheme 2 recorded with a mixing time of 40 ms. (B) Here is an overlay of 1D $^{13}$C spectrum of Aβ$_{40}$ (black online) and Aβ$_{40}$-Cur (red online) showing peaks of two conformations of the $\delta$-carbon of Asp$^{23}$, as indicated. (C) Shown here is an overlay of 1D $^{15}$N spectrum of Aβ$_{40}$ (black online) and Aβ$_{40}$-Cur (red online) showing two conformations of amide $\zeta$-nitrogen of Lys$^{28}$. (D) Here, we probe salt-bridge formation between Asp$^{23}$ and Lys$^{23}$ with fs-REDOR. Dephasing curves for Aβ$_{40}$ and Aβ$_{40}$-Cur aggregates, obtained by monitoring the peak height of the $\gamma$-carbon in Asp$^{23}$ with (S) and without (S0) $^{13}$C-$^{15}$N dipolar recoupling, are shown. (Dotted line) Here is variation of $R_{NC}$ as a function of $t_{mix}$. (Solid squares on top of the dotted line) $R_{NC}$ values of 4.0 and 4.4 Å corresponding to $t_{mix}$ values of 17.1 and 21.2 ms are given. (E) Shown here is an overlay of 2D $^{13}$C-$^{13}$C PARIS-xy $(m=1, N=0.5)$ spectra of 4-day-old aggregates of Aβ$_{40}$-Cur (red online; scheme 2) and 30-min aggregates of Aβ$_{40}$ grown in the presence of curcumin in HEPES buffer (black online; scheme 3), and Aβ$_{40}$ small oligomers (dotted blue online; scheme 2; sample preparation mentioned in (1)) recorded with a mixing time of 40 ms. (Solid arrow) The salt-bridge-forming peaks at 180.1 ppm are missing in 30-min aggregates and small oligomers. To see this figure in color, go online.
us (23) when we incubated 8 μM Zn²⁺ with 10 and 20 μM Aβ₄₀ (23). Reports suggest that the soluble aggregation intermediates, and not the final mature fibrils, are responsible for Aβ toxicity (43–45). Therefore, the sequestration of these species by curcumin and Zn²⁺ is likely the key cause of the reduction of toxicity. We note that our results cannot distinguish which of the aggregation intermediates is the most toxic, because both curcumin and Zn²⁺ precipitate a large range of aggregates.

**Curcumin allows ordered fibril formation**

It is known that Zn²⁺ strongly disrupts fibril formation for both Aβ₄₂ and Aβ₄₀ (23,46). Because Zn²⁺ preferentially interacts near the N-terminus (i.e., with histidine residues 6, 13, and 14), it suggests that this region has a strong role to play in determining the state of the salt bridge, located far away both in sequence and in space (29). On the other hand, Fig. 2 shows that while curcumin shortens the length of Aβ₄₀ fibrils, it does not stop fibril formation altogether. Significantly, it disrupts or reduces the fibril length more in Aβ₄₂ than in Aβ₄₀ (Fig. 2, E and F). Because its major site of interaction with Aβ is at the C-terminal (21), it indicates that the conformation of the C-terminal can also strongly affect the morphology of the fibrils (21,29), and a putative weakening of this interaction in Aβ₄₀ (due to the missing hydrophobic residues I41 and A42) also weakens this effect.

**Oligomers retain their hairpin conformation with both curcumin and Zn²⁺**

Oligomers are characterized by a distal contact formed between F19 and L34, which is retained in the fibril. The row projection of the ssNMR spectra shows the F19-L34 cross peak in Aβ₄₀ incubated with either curcumin (Fig. 3 C) or Zn²⁺ (Fig. 3 D), indicating that the tertiary fold is maintained in the presence of both the reagents. Similar observations were made in the case of Aβ₄₀ earlier (21,29). However, for Aβ₄₀ the cross peaks become relatively weak in aggregates grown in the presence of Zn²⁺, indicating some distortions of the hairpin fold.

**Oligomers are arrested in their non-salt-bridged state by Zn²⁺**

ssNMR observations reported here indicate that the salt bridge between Lys²⁸ and Asp²³ is at least partially disrupted in the Zn²⁺-incubated fibrils of Aβ₄₀ (Fig. 4). Noy et al. (46) have predicted a possible structural perturbation of Aβ₄₀ in the salt-bridge region by Zn²⁺, but the status of the salt bridge was not clear in their study. We have reported...
before that the salt bridge is absent in the Zn$^{2+}$-incubated fibrils of A$\beta_{42}$ (29). While this was interpreted as Zn$^{2+}$ breaking the salt bridge (29), it is possible that Zn$^{2+}$ simply captures A$\beta_{42}$ in an oligomeric state that is yet to evolve a stable salt bridge, and keeps it that way. We note that the presence or absence of the salt bridge in the oligomeric form is not yet established for A$\beta_{42}$. Moreover, recent reports suggest that the terminal A42 and not D23 may form the salt bridge with K28 (8,9). However, in A$\beta_{42}$ containing a C-terminal amide (our preparation), the salt bridge is clearly formed between D23 and K28 (29). This indicates that different fibrillar polymorphs of the natural peptide probably exist, and small changes in the C-terminal may cause large changes in the nature of the salt bridge.

Curcumin allows the salt bridge to form in A$\beta_{40}$

Our results show that the salt bridge is present in the curcumin-incubated fibrils of A$\beta_{40}$ (Fig. 5). This gives rise to two possibilities: either curcumin induces immediate salt-bridge formation in the oligomers, or it allows salt-bridge formation at a later stage, when the fibrils form. To decide between these possibilities, we looked at the A$\beta$ aggregates precipitated by curcumin at an early stage (30 min). We found that the salt-bridge-forming peaks of Asp$^{23}$ are absent at this stage (Fig. 5 E), indicating that curcumin allows salt-bridge formation only at a later stage. However, for A$\beta_{42}$, curcumin does not allow formation of the salt bridge or of well-ordered fibrils even in the later stage.

It is interesting to speculate why curcumin allows A$\beta_{40}$ to form the salt bridge at a later stage of growth. We have found earlier that curcumin interacts strongly with the 34th, 36th, and 38th residues on the C-terminal of A$\beta_{42}$ (21). However, here we do not observe any substantial change induced by curcumin in L34 and G38 of A$\beta_{40}$. This indicates that interaction of A$\beta_{40}$ with curcumin is much weaker than that with A$\beta_{42}$. Two additional hydrophobic residues (I41 and A42) on the C-terminal thus seem to play a crucial role in the way A$\beta$ interacts with curcumin. It is likely that a stronger interaction of curcumin with these extra two amino acids in A$\beta_{42}$ prevents the formation of the salt bridge, but a weaker restriction imposed on the A$\beta_{40}$ molecules allows the aggregation to proceed such that the peptide ultimately forms the salt bridge. We note that it is possible that curcumin has a much lower affinity for the mature aggregates of A$\beta_{40}$ and may tend to dissociate from them as the aggregates mature. In contrast, Zn$^{2+}$ likely interacts with the histidine-containing N-terminal region of A$\beta$ (25–27), which is identical in sequence in both A$\beta_{40}$ and A$\beta_{42}$, and consequently has the same effect on both.

Significantly, the fibril morphology seems to be correlated with the presence of the salt bridge. Zn$^{2+}$-incubated samples never form regular fibrils (23,46), while curcumin-incubated ones do form fibril-like features, though of a shorter length (Fig. 2). It has also been shown by others recently that A$\beta_{40}$ is able to form fibrils in the presence of curcumin (18). There are also reports on other phenolic compounds that can interact with A$\beta_{40}$ in the C-terminal, and still allow salt-bridge formation between D23 and K28 (47).

CONCLUSIONS

We conclude that both Zn$^{2+}$ and curcumin reduce A$\beta$ toxicity by precipitating the non-salt-bridged oligomers. However, curcumin eventually allows the formation of the salt bridge in A$\beta_{40}$, likely due to its weaker C-terminal-mediated interaction with the peptide. This shows the strong effect of the C-terminal residues on the D23-K28 salt bridge, which is far removed from it in the sequence. The effects of Zn$^{2+}$ indicate that the N-terminal also can have a similar effect on the central salt bridge. Thus the N-terminal, the C-terminal, and the central salt bridge containing regions of A$\beta$ appear to have a clear conformational link. This helps to explain several known facts about A$\beta$ toxicity. First, this conformational link suggests that A$\beta_{42}$ can be more toxic than the shorter A$\beta_{40}$ by differently controlling the salt-bridge region, which possibly plays a major role in determining the toxicity of these peptides. It also suggests that most of the mutations leading to early onset AD may actually reflect different facets of a unified toxicity mechanism. In addition, it appears that curcumin preferentially interacts with A$\beta_{40}$ aggregation intermediates, and changes their toxicity—but does not strongly interact with the mature end-product of aggregation. Thus, curcumin can be a starting point for developing a catalytic agent that can modulate the aggregation pathway, reducing toxicity without a net thermodynamic cost.

SUPPORTING MATERIAL

Two figures and one table are available at http://www.biophysj.org/biophys/supplemental/S0006-3495(17)30287-4.

AUTHOR CONTRIBUTIONS

S.M. and P.K.M. designed and conceptualized the experiments. B.C. and V.S.M. performed the NMR experiments and analyzed the data. D.B. performed and analyzed the TEM. A.K.D. performed the cell toxicity assays. B.C. and S.M. cowrote the article. All authors discussed the results and commented on the article.

ACKNOWLEDGMENTS

We acknowledge the National Facility for High-Field NMR and Manoj Naik for assistance in NMR experiments, and the Cryo-TEM facility and Lalit Borde for help with the TEM measurements.

B.C. acknowledges a CSIR-SPM-SRF fellowship.


