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Form and dimensions of aggregates dictate cytotoxicities of Danish dementia peptides

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ABSTRACT

Familial Danish dementia (FDD) is a neurodegenerative disease which results due to alterations in the *BRI2* gene. The pathological symptoms of the disease are cerebral amyloidolysis, parenchymal protein deposits and neuronal degeneration. The ADan peptide is a 34 amino acid long peptide which is thought to be the major cause of amyloid deposition in brains of patients suffering from FDD. Due to the presence of two cysteine residues viz. cys5 and cys23, this peptide exists in two forms: a cyclic oxidized form where the two cysteines form a disulfide bridge and a linear reduced form where the sulphydryl groups of cysteine are free. The relationship between toxicity and structure of the reduced and oxidized forms of ADan peptides has been elucidated by a combination of biophysical and cellular toxicity assays. It is observed that the reduced peptide has a stronger lethal effect on neuronal cell lines compared to its oxidized counterparts at all stages of aggregation. Further, it is observed that the fresh reduced peptide induced greater cell death as compared to its aged counterpart.

Neurodegenerative disorders are characterized by cell/tissue damage caused by the toxic protein aggregates. Anomalous production, processing and clearance of misfolded proteins lead to their agglomeration and eventually to the formation of aggregates [1–3]. The protein folding–misfolding balance is a combined effect of many factors such as hydrophobicity, amino acid sequence, length etc. [4,5]. From the histopathological evidences of the afflicted organ and *in vitro* biochemical and biophysical studies it was proposed that plaques consisting of protein aggregates are crucially responsible for the pathogenesis of these conformational diseases [6,7]. Nonetheless, recent studies describing tissue-specific accumulation of soluble protein oligomers and their strong impact on cell function and signaling point towards a new direction for explaining the pathogenesis in these diseases [8,9]. Both the fibrillar aggregates and the soluble aggregates seem to play different roles on the eventual outcome of the disease.

Familial Danish dementia (FDD) is a neurodegenerative disorder linked to a genetic defect in the *BRI2* gene. It is typified by deafness, cataract, ataxia and dementia. Histological signatures of the disease are cerebral amyloid angiopathy (CAA), parenchymal protein deposits and neurofibrillary degeneration which are analogous to those in Alzheimer's disease (AD) [10]. The ADan peptide is a 34 amino acid long peptide which is thought to be the major cause of amyloid deposition in FDD. Because of the presence of two cysteine residues viz. cys5 and cys23, this peptide exists in two forms: a cyclic oxidized form (oxADan) where the two cysteines form a disulfide bridge and a linear reduced (redADan) form where the sulphydryl groups of cysteine are free. The oxADan peptide is the dominant fiber forming entities at pH 4.8; the redADan peptide is not capable of doing so at any pH and concentrations. Their toxicity depends on the size of their aggregates [10,11].

In this article we report the structure toxicity relationship of the reduced and oxidized ADan peptides. It has been previously shown that the two forms of peptides have different mode of aggregation and final aggregated form in the two are vastly different. They also differ grossly in their toxicities. The result of our study describes in detail the mechanism of aggregation of the two forms of ADan and the relationship between their states of oligomerization and cytotoxicity.

Materials and methods

Preparation of ADan peptide solutions. The reduced and the oxidized form of ADan peptides were bought from Bachem AG (Switzerland) and purified using a C-18 HPLC column in a WATERS-HPLC. The purity and mass of the peptides were assessed by MALDI-TOF mass spectrometer (Bruker Daltonics) in matrix of sinapinic acid containing 0.1% trifluoroacetic acid. The mass of the reduced peptide was found to be 4046.747 Da and that of the oxidized one was found to be 4044.522 Da which are in accordance with previous reports [11]. Prior to the experiments, the peptides

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were subjected to a HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) treatment for disaggregation as mentioned in [11]. Briefly, the peptides were dissolved in HFIP and sonicated in a water bath for 1 h, followed by vortexing for 15 min. This solution was left in HFIP overnight. Finally this solution was lyophilized and then taken for fibrilization. The lyophilized peptides thus obtained were dissolved in a minimum volume of DMSO (dimethyl sulphoxide) under a flow of liquid nitrogen. These were the stocks. In this way peptides can be stored in DMSO for a period of almost a month without any aggregation. All other experiments were performed at pH 4.8 (5 mM sodium acetate buffer, 10 mM sodium chloride, and 0.03% sodium azide) unless otherwise mentioned. Aggregation experiments with the reduced peptides were performed in the presence of 1 mM 1,4-dithiothreitol (DTT). For all experiments the peptide concentration used was 300 μM. The *in vitro* aggregation experiments were carried out at pH 4.8. For the cytotoxicity assay, the aggregates were grown at pH 4.8 buffer without sodium azide and then lyophilized. The lyophilized aggregates were resuspended in culture medium before testing for cytotoxicity. In the case of neurotoxicity assays, the peptides were allowed to aggregate in buffer devoid of sodium azide.

Atomic force microscopy. A drop of ADan peptide solution was placed on a freshly cleaved mica sheet and dried immediately under nitrogen gas. The salt deposits were washed extensively by washing with miliQ water. The samples were once again dried with nitrogen gas. All the images were recorded in air under ambient conditions with a scan rate of 0.3 Hz in a Picoplus (USA) AFM instrument. The resonance frequency used was 75 kHz.

Cell neurotoxicity assay. The effect of ADan peptides on cell viability was assessed by measuring residual cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) as previously described [11]. Briefly, SHSY5Y cells (in RPMI medium with 5% FBS) were plated at 10^5 cells/well in 96-well plates in 90 μl of fresh medium. The peptides were then re-suspended in fresh medium and added to the cells in the plate. Cells were then incubated at 37 °C in 5% CO_2 for 24 h, following which MTT was added to a final concentration of 0.5 mg/ml and the plates were incubated at 37 °C for 3.0 h. The formazan formed was solubilized in isopropyl alcohol (150 μL) and incubated for three hours at 37 °C. Finally, the absorbance values at 570 nm were then determined. DTT has no effect on the cell viability; hence the toxicity assay for the reduced peptide was carried out in the presence of 1 mM DTT.

Fourier transform infrared spectroscopy. IR spectra were collected in a Bruker Tensor 27 FTIR system equipped with a liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector, at a nominal resolution of 2 cm^{-1} in both Aquaspec cell and BioATR mode. Briefly, the peptide solutions were placed in between the CaF_2 windows of the Aquaspec cell and 256 spectral scans in the range 1700–1600 cm^{-1} were obtained. All spectra were vector normalized in the above spectral region. Data analysis was performed with the OPUS software provided with the instrument. Similar approach was used to collect data in the BioATR. The buffer spectrum was each time subtracted from the final spectra.

Results

Aggregation in the oxADan and redADan

The process of aggregation of both oxidized and reduced forms of the peptide have similar initiation profiles as evident form the Infrared spectra and atomic force microscopic images. As observed by AFM, at a concentration of 300 μM, both the forms lead to the formation of small protofibils by 12 h (Fig. 1). After 12 h, the protofibrils of oxADan associate with one another leading to the formation of fibers. A few fibrillar strings can be seen after a period of 24 h. At this time point a mixture of protofibrillar species along with budding fibers is seen (Fig. 1b). This process of association is then further carried on and the amount of protofibers in the ensemble is found to decrease substantially by the end of 72 h with a concomitant increase in the density of fibers (Fig. 1c). After 120 h there is a substantial increase in the length of the fibers (Fig. 1d). In the case of redADan, protofibers are formed after 12 h of incubation (Fig. 2a). However, after this stage the features of maturation of the aggregates deviate from the oxidized form. The redADan protofibrillar aggregates further associate together in a non-specific manner to form larger clumps of no specific structure and form. The aggregates formed by the reduced peptide after 72 h of incubation as shown in Fig. 2b has no particular pattern of organization. Neither intermolecular nor intramolecular disulfide bonds are formed in the redADan peptide when incubated for a week in the presence and absence of 1 mM DTT, as evident from the appearance of a consistent peak at 4046.7 Da in the MALDI-TOF mass spectrum (Figs. S1 and S2, Supplementary Information). Thus the redADan retains its two free cysteines under experimental conditions.

FTIR profiles for the aggregation of the two forms of ADan also vary considerably with time (Fig. 3). In both the cases, initiation of the aggregation process is similar with the appearance of the beta sheet peak (at 1636 cm^{-1}) and simultaneous development of the cross beta sheet peak (at 1685 cm^{-1}) [12–14], but the pattern of their propagation is not the same. The increase in the peak area for both the beta sheet and cross beta peak is monotonous for the oxidized form, whereas randomness is observed for the reduced form. This shows that the fibrillar form of oxADan as seen by AFM is a result of formation of cross beta sheet stabilized structures. That the disulfide bond aids in the formation of fibers has also been shown by Das et al., for a modeled peptide system [15].

Toxicity of the oxADan and redADan

Cytotoxicity experiments were performed with both the forms of the peptides at different stages of their aggregation. While redADan does not undergo disulfide bond formation spontaneously 1 mM DTT which by itself is not cytotoxic to neuronal cells was included in the cytotoxicity assay to forestall even the chance formation of a disulfide bond in redADan. It is observed that the fresh peptide in both the forms (soluble and non-aggregated) is more toxic compared to their completely aggregated counterparts. In fact the small spherical protofibrillar masses are also more toxic compared to the completely aggregated fibers. Comparing the oxidized and the reduced peptides we note that the redADan peptide is more toxic than its oxidized countepart (oxADan) at all stages of aggregation. Thus, for the fresh peptides the lethal activity for the redADan starts as soon as the peptide is added to the culture and lethality to the extent of 40% is observed at the end of three hours with marked change in the morphology of the cells (Fig. 4A). For the oxidized form the viability of the cells is almost similar to the untreated control cells at 3 h. Moreover the cells also retain their morphology (Fig. S3a). However, after 6 h of incubation with oxADan, mild changes in the cellular morphology occur along with death of some cells. Overall the viability of the cells treated with redADan and oxADan after 6 h is 42% and 67%, respectively. The fresh redADan shows 30% cell viability after 24 h of incubation, while oxADan shows 60% cell viability under identical conditions (Fig. 4A).

However the lethality on the neuronal cells is reduced markedly when the cells are treated with the peptides aged for 72 h. Thus at 72 h redADan which forms spherical aggregates induces only ~19% cell death (Fig. 4B).

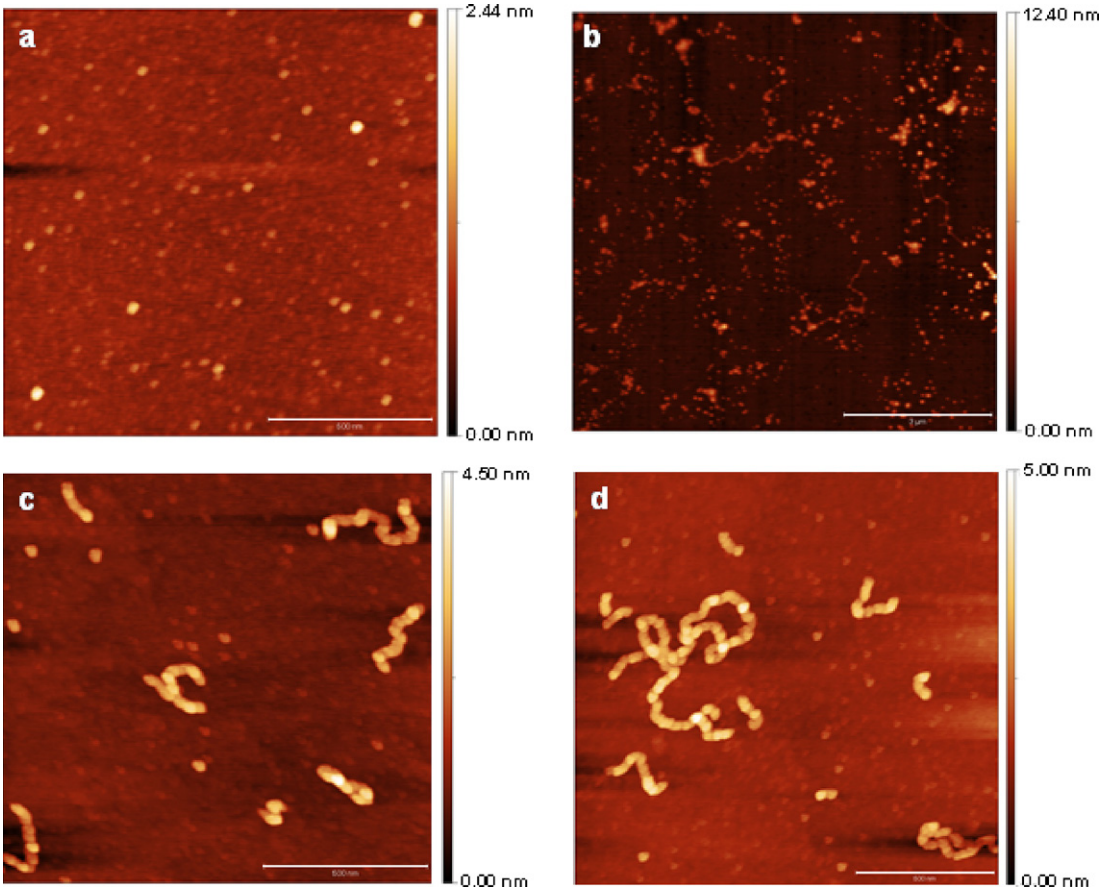

Fig. 1. Atomic force microscopy images of different stages of aggregation of oxADan peptides. (a) oxADan incubated for 12 h shows the formation of spherical protofibrils (scale 500 nm). (b) 1-day-aged oxADan showing both protofibrils and buddung fibers (scale 1 micron) (c) 3 days old oxADan (scale 500 nm). (d) 5 days old oxADan [scale 500 nm].

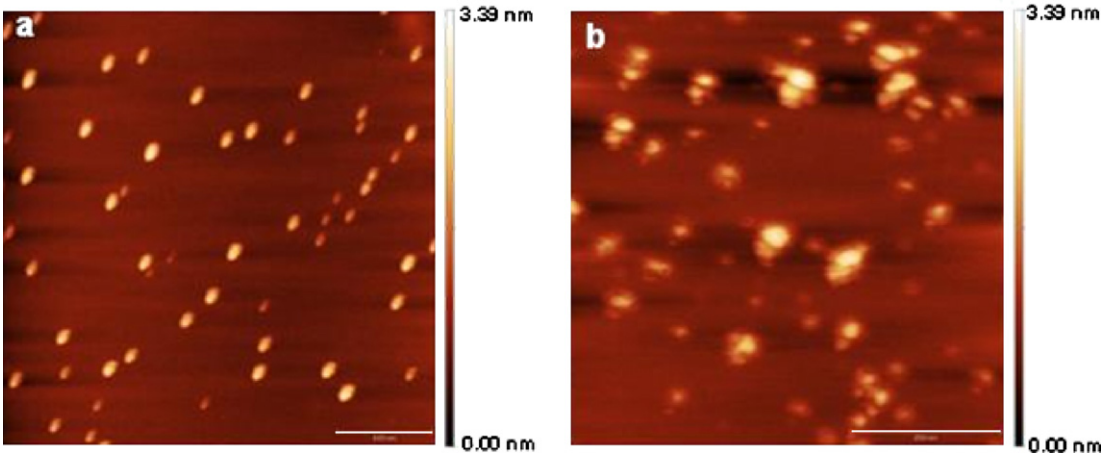

Fig. 2. Atomic force microscopy images of different stages of aggregation of redADan peptides. (a) redADan incubated for 12 h shows the formation of spherical protofibrils (scale 500 nm). (b) 3 day aged redADan (scale 200 nm).

Both redADan and oxADan exhibit completely aggregated forms by 120 h. The completely aggregated forms of redADan and oxADan, however, give a very different picture. The toxicity effects of both the forms are almost similar at all time points of observation. After three hours of incubation of the cells with the aggregates of either redADan or oxADan, there is negligible loss in viability; 25–30% cell death occurs after 6 h of incubation and ~40% of cell death upon incubation for 24 h (Fig. 4C) with fully formed aggregated fibers of the ADan peptide.

Discussions

It has been shown that the amyloidogenic proteins and peptides can adopt a host of different aggregation states under different sets of conditions. Recently, consensus is emerging that shows that the fibrillar aggregates are not the predominant species responsible for the pathogenesis of the disease and perhaps small soluble oligomers are the crucial players for the eventual cytotoxicities exhibited by them [9,16,17]. However, the direct relationship of the

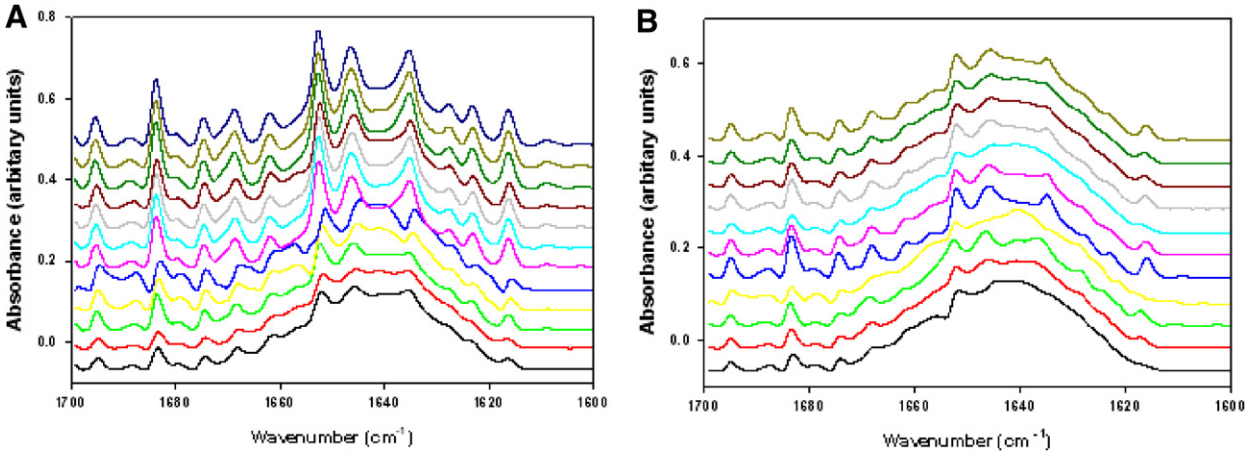

Fig. 3. The course of maturation of oxADan and redADan aggregates as observed by FTIR spectroscopy. (A) oxADan. (B) redADan.

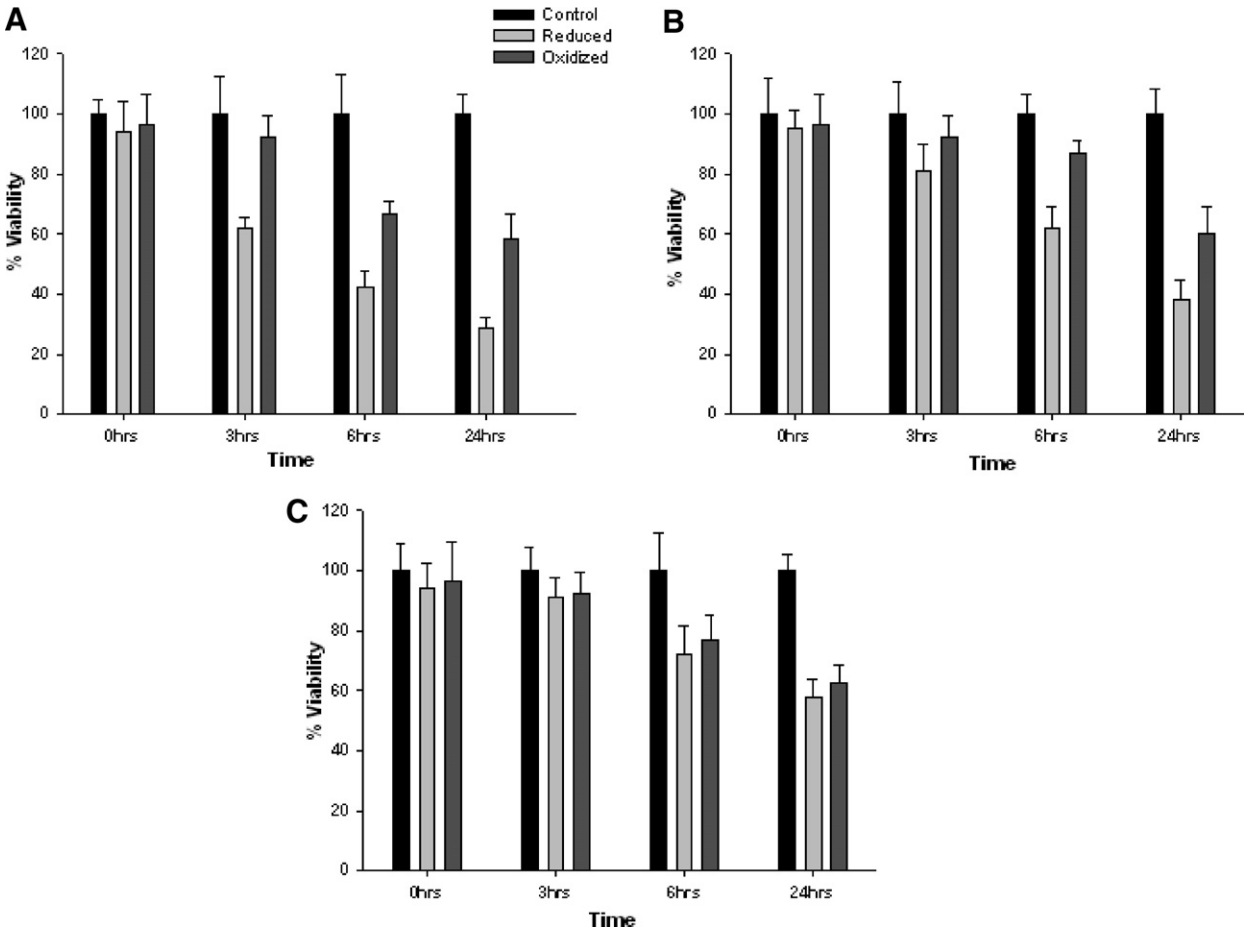

Fig. 4. MTT assay performed on the SHSY5Y cells with (A) Fresh solutions consisting of either redADan or oxADan. (B) Cells treated with redADan or oxADan for 72 h and (C) cells treated with redADan or oxADan aged for 120 h. Experiments with reduced peptide were carried out in the presence of 1 mM DTT, as it was found to have no effect on cell viability. Cells not treated with peptides are referred to as controls.

various intermediates of ADan peptide with cytotoxicity or neurotoxicity has not been established.

In the case of ADan peptides the issue of cytotoxicity is quite perplexing. The two forms of the peptide exhibit different extents of lethality under various conditions. Cell mortality here is a function of two things: the form of the peptide and its aggregation state. The main observations from the present studies are that the soluble oligomers of redADan are the most lethal entities and that as the size of the aggregates increases their toxicity declines progressively. This clearly suggests that toxicity has a definite relationship with the valency of the aggregating sites on the oligomerizing species under consideration. With maturation the valency or the interacting sites on the peptide molecule get saturated which in turn decreases the lethality of the peptide.

Comparing the toxicity of the reduced and the oxidized ADan peptides, it is observed that while in the case of the former the extent of cell death depends on the size and aging time of the aggregates, the later ones present almost a constant pattern of lethality with the dif-

ferent types of aggregates under discussion. For the soluble aggregates of reduced peptides as discussed above the cell death ranges from 70% to 19% for the different forms of the aggregate. The oxADan on the other hand exhibits 40% less toxicity as compared to redADan at all the corresponding time points. However, the differences in their lethalities are broadly similar for their fully aggregated forms.

A precise examination of the FTIR spectra show that all the genre of the two classes of peptides have almost similar pattern as far as the profiles of the initiation reaction is concerned. There is an increase in the intensity of the peak at 1634 cm^{-1} in both the forms, which corresponds to the formation of β-sheet structure. The β-sheet formation for the oxidized peptide shows a monotonous increase with time along with the simultaneous appearance of the intermolecular β-sheet at ~1685 cm^{-1}. The enhancement in peak intensity for the β-sheet and the cross β-sheet are at par with each other for oxADan. This is not pattern reflected in the FTIR spectra for the redADan. At the initial phase there are signatures for the formation of the β-sheet structure. But the emergence of the β-sheet as well as the cross β-sheet structure does not follow any particular pattern. In fact, there is a high degree of randomness in the formation of aggregates in redADan. While at some time points one does see β-sheet and cross β-sheet signatures, but this pattern is not conserved over different sets of experiments.

The sequential mode of fibrillogenesis in oxADan and the randomness in the aggregation in redADan is also reflected in the AFM images. Although the initial modes of association in both of them are similar, they take up different pathways for maturation. As reported by us earlier, the oxADan undergoes a hierarchical mode of fibrillation by the sequential formation of protofibrils, protofilaments, and mature fibers [11]. In the present study this is further confirmed by AFM and FTIR. Figs. 1 and 2 show the different phases of aggregation of the oxADan and redADan. The monomers of redADan peptides associate very fast as reflected by the appearance of ~2–3 nm spherical protofibers after only 12 h of initiation of the reaction. In the case of the oxADan, these protofibers associate in a way so as to form long slender filamentous aggregates and eventually form fibers. On the other hand, the redADan cannot associate like the former to give rise to fibrillar entities. This is a sort of non-specific aggregation where the protofibers clump together in a variety of ways to form aggregates of no specific form and size. Interestingly, however, the reduced peptides are found to be more toxic than the oxADan. Aggregated redADan as well as its fresh form are more toxic than any forms of the oxidized peptide as evident from the change in morphology of neuronal cell lines upon incubation with these peptides (Fig. S3).

Formation of the disulfide bond in ADan peptide therefore does not dictate the formation of the seeding entities for fibrillogenesis and therefore must be considered to contribute minimally to the toxic pathology of this peptide. However, it has a crucial role to play in the final destiny of the aggregates. The cyclic oxidized peptide renders itself in a conformation that promotes fiber growth. The linear reduced form due to its extended valency looses specificity of the interactions required for aggregation and start to interact in a somewhat haphazard manner. These studies thus emphasize the importance of the disulfide bond in these peptides in the course of events which eventually has a bearing on the cytotoxic events brought about by them and the fibrillogenesis. While the proper orientation of the disulfide is not required for the initiation of the process, it is required for the appropriate progression for fibril formation. Coming to the issue of toxicity, our studies show explicitly that the morphology of the aggregates has an important role in defining the toxicity of the species under question. This is in qualitative agreement with a recent report by Yoshiike et al., where the authors have shown that surface structures of the amyloid β-fibrils are the main players in determining the toxicity of the species under question [18].

There are a number interesting aspects of the present work not highlighted earlier. Firstly, this work demonstrates that the toxicity of the redADan is much more than that of its oxidized counterpart, oxADan. Secondly we have shown that the fresh redADan is more toxic than its aged counterpart. The progress curve of aggregation and the nature of the aggregated products are not the same in the case of the oxidized and the reduced form of ADan peptides. Another novel finding of these studies is the observation that the toxicity of the reduced peptide, redADan varies considerably with the size and age of its aggregates, while, that of the oxidized peptide remains constant for most of the stages of its aggregation and finally this article demonstrates both spectroscopically and by atomic force microscopy the differences in the itinerary of aging pathway of the two peptides and attempts to provide a rationale behind it.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.169.

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