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Methionine oxidation reduces lag-times for Amyloid-β(1-40) fibre formation but generates highly fragmented fibres

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Running Title: Methionine oxidation and Amyloid-β fibre formation

Abbreviations:

Alzheimer's disease, AD; Amyloid-beta peptide, Aβ; Methionine sulphoxide, Met^{ox}; Methionine sulphoxide reductase, Msr; Transmission Electron Microscopy, TEM; Thioflavin T, ThT.

Abstract

Oxidative stress and the formation of amyloid plaques containing Amyloid-β (Aβ) peptides, are two key hallmarks of Alzheimer's disease. A proportion of methionine (Met) at position 35 within Aβ is oxidized to methionine sulphoxide (Met^{OX}) within the Alzheimer's plaques. These oxidative processes may be the key to understanding the early stages of Alzheimer's disease. *In vitro* oxidation of Aβ, by the physiological oxidant H_2O_2, was monitored using ^1H NMR and mass spectrometry. Here we investigate the effect of Aβ methionine oxidation on fibre formation kinetics and morphology using the amyloid specific fluorescence dye Thioflavin T (ThT) and Transmission Electron Microscopy (TEM). Methionine oxidation reduces the total amount of fibres generated for both dominant forms of Aβ, however there are marked differences in the effect of Met^{OX} between Aβ(1-40) and Aβ(1-42). Surprisingly the presence of Met^{OX} reduces lag-times for Aβ(1-40) fibre formation but extends lag-times for Aβ(1-42). TEM indicates a change in fibre morphology with a pronounced reduction in fibre length for both methionine oxidized Aβ(1-40) and Aβ(1-42). In contrast, the morphology of preformed amyloid fibres are largely unaffected by the presence of H_2O_2. Our studies suggest methionine oxidation promotes highly fragmented fibre assemblies of Aβ. Oxidative stress associated with Alzheimer's Disease can cause oxidation of methionine within Aβ and this in turn will influence the complex assembly of Aβ monomer into amyloid fibres, which is likely to impact Aβ toxicity.

Key word: Methionine; Oxidation; Amyloid; Alzheimer's; Fibre; Kinetics;

Highlights

Methionine oxidation (Met^{OX}) reduces the total amount of Aβ fibres generated

Met^{OX} reduces lag-times for Aβ(1-40) fibre formation but extends lag-times for Aβ(1-42)

Met^{OX} perturbs amyloid structure and causes a marked reduction in Aβ fibre length

Preformed Aβ amyloid fibres are largely unaffected by hydrogen peroxide

Met^{OX} promotes highly fragmented fibre assemblies of Aβ

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and directly affects more than 30 million people worldwide [1]. Central to the amyloid cascade hypothesis in AD is the accumulation of amyloid-β peptide (Aβ) into plaques [2]. These plaques are dominated by two forms of Aβ, forty (1-40) and forty-two (1-42) amino acids long. Aβ(1-40) is more abundant but Aβ(1-42) is thought to be more toxic and forms amyloid fibers more readily. Another key hallmark in AD patients are numerous markers of oxidative stress [3] early in the amyloid cascade [4, 5]. Oxidative stress is characterized by an imbalance in reactive oxygen species (ROS) and anti-oxidative defense, which leads to neurodegeneration and cognitive decline [6]. Studies have shown evidence of increased levels of oxidative damage in the brain tissue, as well as in the cerebrospinal fluid (CSF) and plasma, of patients with AD [3]. Observed oxidative damage includes protein oxidation [7], DNA and RNA oxidation [8] and also lipid peroxidation [9].

The Aβ peptide can be influenced by oxidative processes. Several residues within Aβ including, Tyrosine and Histidine have been shown to be oxidized within plaques [3, 10-13] while the N-terminus can be cyclized through the formation of a α-ketoamide [14]. Methionine is particularly prone to oxidation and it is well established that a proportion of Aβ has its single methionine (residue 35) oxidized to a sulphoxide in the plaques of patients' brains *ex vivo* [15, 16]. Chemical analysis of amyloid plaques from transgenic mice models of AD, also report Aβ with methionine-sulphoxide [17]. Importantly, a decline in the activity of the methionine sulphoxide reductase enzyme (Msr) is observed in AD patients [18] and the Msr gene has been shown to modulate Aβ aggregation in a *C. elegans* model of Alzheimer's [19]. This raises the question of whether the extent of methionine oxidation influences the complex assembly pathway of Aβ oligomers, fibres and plaques.

As one of the most readily oxidized amino acid residues within Aβ, Met35 can be oxidized by hydrogen peroxide, hydroxyl radicals, hypochlorite, chloramines or peroxynitrite. Although less reactive compared to other reactive oxygen species, H_2O_2 oxidizes methionine without the presence of oxidative free-radicals [20]. H_2O_2 is generated by the dismutation of the superoxide radical and is a ubiquitous molecule *in vivo* [21]. Cu^{2+} bound Aβ is concentrated in plaques and [15, 22-24], when bound to Aβ remain redox active, readily generating hydroxyl radicals and H_2O_2 in the presence of a physiological reductant such as ascorbate [12, 25-31]. This will result in oxidation of Histidine [12, 13], Tyrosine [10, 11] and particularly Methionine within Aβ [15-17].

In Alzheimer's disease oxidative stress can occur before the appearance of amyloid plaques [4, 5]. However, it is not yet clear if methionine oxidation is a consequence or a trigger for Aβ plaque formation *in vivo*. Despite the presence of Met^{OX} in Aβ plaques there have been surprisingly few detailed studies on the influence of methionine oxidation on the kinetics of Aβ fibre formation. We are aware of only one study that directly monitors the kinetics of Aβ(1-42)Met^{OX} fibre formation [32]. This study used the binding of Thioflavin T (ThT) to monitor fibre growth *in vitro* and indicated that Met^{ox} inhibits amyloid fibre formation of Aβ(1-42) [32]. Although not directly monitoring the kinetics of fibre formation, the disrupting effect of Met^{OX} on β-sheet formation was also described for Aβ(1-40) using circular dichroism [33]. Solution NMR studies suggest that Met^{OX} prevents β-sheet production by reducing both hydrophobic and electrostatic associations during the initial stages of Aβ(1-40) and Aβ(1-42) aggregation [34]. Interestingly, the oxidation of Met35 in Aβ(1-42) has been shown to block paranucleus formation, which is characteristic of Aβ(1-42), and produced Aβ(1-42)Met^{OX} oligomers indistinguishable in size and morphology from those produced by Aβ(1-40) [35]. Mass spectrometry shows Met^{OX} inhibits trimer but not dimer formation in the early rate-limiting step of Aβ(1-40) aggregation [36]. In contrast, an early study using sedimentation measurements suggested Met^{OX} enhances the precipitation rate of Aβ(1-40), however these studies made no distinction between amorphous aggregates and ordered fibres [37]. Studies of senile plaques isolated from the brains of AD patients indicate changes in fibre morphology upon Met35 oxidation of pre-existing amyloid fibres [38].

Reports on AβMet^{OX} dependent cell toxicity are rather mixed, some cell viability experiments studies suggest that Met oxidation increases the toxicity of preformed mature Aβ(1-40) and Aβ(1-42) fibres [39] and the anti-oxidant enzyme catalase protects cells from Aβ toxicity [28, 40, 41]. On the other hand, more recent studies suggest Met oxidation of Aβ reduces cell toxicity [42-45], by upregulating methionine sulphoxide reductase (Msr) [46]. Furthermore, only Met35 in a reduced form will disrupt Ca^{2+} homeostasis in neuroblastoma cells [47] and disrupt synaptic function [48].

The consensus in the literature suggests Met^{OX} of Aβ(1-40) and Aβ(1-40) inhibits the formation of fibres [32-34], yet plaques contain Met^{OX} [15-17] and AD is characterised by a reduction Mrs activity [18, 19]. For this reason we wanted to revisit the influence of Met^{OX} on fibre formation. It is important to establish if Met^{OX} might be a trigger for fibre formation or simply a consequence of plaque formation. Furthermore, although Met^{OX} is generally thought to inhibit fibre formation [32-34] little is known regarding the structural impact of Met^{OX} on the Aβ assemblies generated [49]. Here we aim to investigate the influence of methionine oxidation on both Aβ(1-40) and Aβ(1-42) fibre formation kinetics and its impact on the morphology of Aβ fibres *in vitro.*

2. MATERIALS AND METHODS

2.1 Solubilisation of Amyloid-beta

Amyloid-β(1-40) and Aβ(1-42) (from Cambridge Research Biolabs) were dissolved at 0.7 mg/mL pH 10.5, kept at 4 °C with gentle rocking for a 12 hours. This protocol has been found to be an effective solubilization protocol [50, 51]. This generated predominantly seed-free monomeric Aβ stock, based on a clear lag-phase observed via ThT fluorescence. Also, a lack of detectable assembles in TEM images and a single elusion peak in size exclusion chromatography indicated seed-free Aβ. Other chemicals were purchased from Sigma.

2.2 Fibre Growth Assay

Fibres were generated by incubation of Aβ(1-40) or Aβ(1-42) at 10 μM, 30 °C in 100 mM HEPES buffer at pH 7.4 with 160 mM NaCl and 20 μM Thioflavin (ThT). BMG-Galaxy and Omega fluoro-star fluorescence 96-well plate readers were used to monitor fibre formation with mild agitation (45 or 60 seconds every 30 minutes). The binding of ThT to amyloid fibres was used to monitor the kinetics of Aβ fibre growth. When bound to amyloid fibres, ThT fluoresces at a maxima of 489 nm, the intensity of which is directly related to the concentration of Aβ fibres present [52]. By exciting at 440 nm and measuring the fluorescence at 490 nm, fibre formation can be followed over time [53]. Although hydrophobic pockets in proteins can induce mild fluorescence of ThT, the fluorescence when bound to in-register stacked hydrophobic residues in fibres is very intense. This fluorescence is caused when the aromatic rings within ThT can no longer freely rotate relative to each other [54]. Cautious interpretation of ThT fluorescence as a quantitative measure of fibre mass is needed [53], as not all Aβ assemblies fluoresce with ThT. In particular, the ThT fluorescence with prefibrillar oligomers and protofibrils of Aβ are typically very weak [52]. Furthermore it may be possible that the intensity of fluorescence may vary slightly for different fibre morphologies.

Conversion of Aβ monomer to fibre follows a characteristic sigmoidal fibre growth curve, which has a lag-phase (nucleation) and a growth-phase (elongation). The lag-phase involves the formation of an increasing number of small nucleating assemblies. The number of individual assemblies (rather than assembly mass) can increase by both primary and secondary nucleation and also fragmentation. The growth-phase (elongation) is dominated by the addition of Aβ monomers on to the ends of growing fibres which leads to rapid increases in fibre mass (and ThT fluorescence) [53]. Some important empirical parameters were obtained from the fibre growth curves, including the time needed to reach half-maximal ThT intensity (t_{50}), the apparent fibre elongation rate (k_{app}) and the lag-time to nucleate fibres (t_{lag}). These values were extracted from the data by fitting the growth curve to the following equation [55].

$$Y = (y_i + m_i x) + \frac{\upsilon_f + m_f x}{1 + e^{-(\frac{x - xo}{\tau})}}$$

Where y is the ThT fluorescence intensity, x is the time and x_o is the time at which the ThT fluorescence has reached half maximal intensity referred to as t_{50}. y_i and υ_f are the initial and final fluorescence signals. The apparent fibre growth (elongation) rate (k_{app}) is calculated from, $k_{app}=1/\tau$ and the lag-time (t_{lag}) is taken from, $t_{lag}= X_o -2\tau$ [55].

The reciprocal of lag-time (t_{lag}) is largely influenced by the microscopic rate constants associated with a combination of primary and secondary nucleation as well as fragmentation. While the apparent fibre growth rate (k_{app}) is influenced by the elongation rate constant, due to monomer addition on to a growing fibre [53]. Determination of microscopic processes that more directly correspond to physical properties of the system such as the numerus rate constants for individual reactions require a global fit of the reaction kinetics data using multiple concentrations of Aβ [53] and are beyond the scope of the current study.

Empirical kinetic parameters have been extracted from between 4 and 12 individual kinetic traces. Analysis of variance (ANOVA) was used to confirm significance in the differences between the kinetics of fibre formation under various concentrations of H_2O_2. A one-way ANOVA with Tukey's HSD post-hoc test was used to reveal significant differences with p values of < 0.05.

2.3 Transmission Electron Microscopy (TEM)

Glow-discharged carbon-coated 300-mesh copper grids, purchased from SPI, were prepared using the droplet method, where 10 µl aliquots of samples from the fibre growth assay were absorbed for 1 min and blotted with filter paper. After rinsing with deionized water (10 µl for 1 minute) and blotting, samples were placed onto a drop of 2.0% phosphotungstic acid (PTA) (purchased from Sigma), pH 7.4 (10 µl for 1 minute), blotted, rinsed and air-dried. Images were recorded on a JEOL JEM-1230 electron microscope operated at 80 keV.

2.4 ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectra were obtained using a Bruker Avance spectrometer, operating at a 600 MHz ^{1}H frequency. Water was suppressed using the excitation sculpting with gradients method. NMR samples contained 10 µM Aβ (1-40) with varying amounts of H_2O_2, the pH of NMR samples were recorded before and after acquisition. Spectra were recorded at 30°C, pH 7.4 in 100% D_2O with 100 mM sodium phosphate buffer. An exponential line broadening of 3.0 Hz was applied to the data before Fourier transformation. Chemical shifts were referenced to water, or formate, which produces a singlet at 8.495 ppm.

2.5 Mass Spectrometry (MS)

Electrospray Ionisation Mass spectra (ESI-MS) were recorded with Aβ(1-40) (10 μM) incubated, with or without, 500 μM H_2O_2 for 7 hours in water. Samples were then diluted into 30% acetonitrile/0.1% (v/v) formic acid before introduction into the ESI-MS, Synapt HDMS System (Waters Corporation, Milford, MA, USA). The time of flight mass analyser was tuned in V-mode to give an operating resolution of 7,000 (Full Width Half Maximum).

2.6 Conformational Antibody Dot Blot Assay: Aβ Oligomer-A11 and Aβ fibre antibody-OC

The Dot Blot Assay was performed as previously described [56] 10 μM Aβ was incubated with and without the presence of H_2O_2, then 6 μl of sample was added on to a Hybond-ECL nitrocellulose membrane (purchased from Amersham Biosciences, Piscataway, NJ, USA). After 10 minutes air dry, the membrane was blocked in 10% nonfat milk in TBST buffer for 3 hours at 4°C [containing NaCl, Tris, and Tween 20 as per Invitrogen (Carlsbad, CA, USA) protocol]. Then, the membrane was washed 3 times with TBST buffer and incubated in 0.5 mg/ml unconjugated rabbit (polyclonal) primary antibody in 5% nonfat milk in TBST buffer at 25°C for 1 hour. After being washed, the membrane was then incubated in the enzyme conjugate (F(ab')2 fragment of goat anti-rabbit IgG (H+L), alkaline phosphatase conjugate, (F21456; Invitrogen) for 1 hour at room temperature. Finally, the membrane was washed once and then incubated in NBT-BCIP substrate solution (BCBH8024V; Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. Stained dots is indicative of the presence of oligomeric species, but not Aβ monomer or fiber. Primary antibody includes A11 and OC antibody. Positive A11 (A11; cat. no. AHB0052; Invitrogen) staining is indicative of the presence of oligomeric species, but not Aβ monomer or fibre [57] while the OC antibody is indicative of Aβ amyloid fibres only.

3. RESULTS

3.1 H_2O_2 causes Methionine to form Methionine sulphoxide.

In vivo Aβ is exposed to oxidants that cause Met35 to be oxidized. In vitro we chose to use H_2O_2, which is a relatively mild oxidant present physiologically, to oxidize methionine to Met^{OX} within Aβ. H_2O_2 was added to Aβ over a range of concentrations (between 0.1 mM and 5 mM) using the same conditions subsequently used to study amyloid fibre formation kinetics and structure.

^{1}H NMR and mass spectrometry was used to monitor oxidation of Aβ under conditions used in our fibre growth studies. Figure 1 shows the aliphatic region of the ^{1}H NMR spectrum of Aβ(1-40). Upon the addition of 0.5 mM H_2O_2, a singlet peak at 2.1 ppm assigned to ϵCH_3 of the single Methionine in Aβ reduces in intensity with time and a new peak of similar intensity appears at 2.7 ppm, which is assigned to the ϵCH_3 of Met^{OX} [12, 58]. After 20 hours, the Met

ϵCH_3 peak at 2.1 ppm completely disappears and is replaced by a peak at 2.7 ppm. We repeated this experiment with different concentrations of H_2O_2 between 0.1 mM and 5 mM. Table 1 shows the time for a 50% reduction in the intensity of the peak at 2.1 ppm for Met ϵCH_3 with different concentrations of H_2O_2. It is clear that increasing the concentration of H_2O_2 increases the rate of methionine oxidation to Met^{OX}. Depending on the level of H_2O_2 there was a partial or more complete oxidation of methionine within the time scales used in the subsequent fibre growth experiments. For example, in the first 100 hours, which is the typical lag-time for Aβ(1-40) fibres to form, 100 μM H_2O_2 will only oxidize a small amount, (*ca.* 30%) of Met35, while 300 μM will oxidize almost all (*ca.* 90%) of the Met35 within Aβ.

Inspection of the ^{1}H NMR spectra suggests that under these conditions only the single Met35 residue is oxidized while other residues, particularly His and Try side-chains which are prone to oxidation, are unaffected. Mass spectra analysis of Aβ(1-40) treated with 0.5 mM H_2O_2 for 7 hrs indicates an increase in the mass by a single oxygen (16 Da), shown in Figure 2, this is consistent with oxidation of methionine to Met^{OX} . There is also a weak additional mass (32 Da) associated and is attributed to further oxidation of the methionine to sulphone, Figure 2.

3.2 Effect of H_2O_2 induced methionine oxidation on Aβ fibre growth

Assembly of Aβ into oligomers and fibres is fundamental to the etiology of AD, in particular, the accelerated rates of Aβ fibre formation *in vitro* correlates with disease onset in familial AD. We were therefore interested in how methionine oxidation might affect the Aβ fibre growth kinetics. We used a well-established 96-well plate assay in which the binding of ThT to Aβ fibres induces a fluorescent signal, which is used to monitor the kinetics of fibre formation [52] [53]. Figure 3 shows Aβ(1-40) fibre formation, monitored by ThT in the presence and absence of 300 μM H_2O_2. Met oxidation clearly influences fibre formation, with lag-times reducing from 180 ± 18 to 131 ± 24 hours. Perhaps counter intuitively, although the nucleation phase is accelerated, the total amount of ThT binding fibres is reduced, as indicated by the maximum ThT fluorescence observed. This is also supported by a reduction in the total amount of long amyloid fibres observed in TEM images. It is notable that fibre dependent ThT fluorescence produced from preformed fibres (discussed later) is unaffected by H_2O_2. The reduction in the ThT signal observed cannot be due to H_2O_2 directly oxidizing the fluorescent dye but is instead is predominately caused by a reduction in the total amount of fibres present.

Others have not reported AβMet^{OX} exhibiting a reduction in lag-times [32], so to assess more fully the effect of H_2O_2 oxidation on Aβ fibre growth, we generated fibres over a range of H_2O_2 concentrations, shown in Figure 4 and supplemental Figure S1. These experiments support the observation made in Figure 3. For example, 100 μM H_2O_2 decreases lag-times by only one quarter, but when 500 μM H_2O_2 is used the lag-times (t_{lag}) are halved. Although nucleation-

phase of fibre formation are accelerated, the total amounts of fibres formed are reduced. Furthermore, the apparent rates of elongation (k_{app}) are reduced, for example, fibre elongation rates are 5 times slower with Aβ(1-40) oxidized by 300 μM H_2O_2, Figure 4a. Statistical analysis, ANOVA, of 6-8 individual traces indicates that the trends in t_{lag}, k_{app} and fibre maximum are significant (P> 0.05) in the presence of 100 μM H_2O_2 and above, Figure 4. It should be appreciated that from our studies of methionine oxidation rates (Table 1), in the presence of 100 μM H_2O_2 only half of the methionine has been oxidized (at the time of fibre nucleation) while with 200 or 300 μM H_2O_2 most of the Methionine will be oxidized. This explains why the effects on fibre formation are more marked at the high H_2O_2 concentrations. We chose this simple method of oxidation to reflect the complex processes that must occur *in vivo*, in which Aβ will be present in both oxidized and reduced forms during the process of fibre assembly.

Next we investigated the effect of H_2O_2 oxidation on Aβ(1-42) fibre formation, shown in Figure 5. As with Aβ(1-40) the total amount of fibres formed is reduced by increasing methionine oxidation. The ThT fluorescence signal is reduced by 70% with 500 μM H_2O_2 added. However, in marked contrast to Aβ(1-40) the lag-times t_{lag} extended from 21 to 144 hrs. At higher levels of H_2O_2, 1000 μM, little fibre formation is detected by ThT fluorescence.

3.3 Influence of Methionine Oxidation on Fibre Morphology

In order to investigate the effect of Met35 oxidation on fibre morphology, we use TEM to study Aβ(1-40) samples incubated with various H_2O_2 concentration under conditions that would otherwise typically have generated mature amyloid fibres. Figure 6 shows TEM images of Aβ(1-40) fibres formed without H_2O_2 or in the presence of 100, 300 and 500 μM H_2O_2 after 400 hrs of incubation. -

In the absence of H_2O_2, the mature fibres are typically long and unbranched structures 10-20 nm thick and many microns in length, Figure 6a. The co-occurrence of short fibres and mature fibres was frequently observed with low concentration of H_2O_2, 100 μM (Figure 5b). TEM images of Aβ incubated with 300 and 500 μM H_2O_2, Figure 6c and 6d, indicate less frequently observed assemblies consisting of much shorter fibres, typically less than 500 nm in length. In addition, spherical oligomers, 10-20 nm in diameter, were observed. These observations were consistently made over a number of preparations and TEM grids. The reduction in fibre content agrees with our ThT fluorescence measurements (Figure 3 and 4) that show a reduction in the total ThT fluorescence of typically 50%.

Negative stain TEM images were also obtained for Aβ(1-42) incubated with H_2O_2 for 240 hours, shown in Figure 7. With 500 μM H_2O_2 a marked reduction in the number of fibres is observed on the TEM grids, although some micron long fibres are still observed. Incubation of

Aβ(1-42) with 1000 μM H_2O_2 for 10 days results in exclusively fragmented short amyloids fibres typically 200-500 nm long and rarely longer, as shown in Figure 7b.

We were interested in further characterizing the assemblies generated in oxidizing conditions, 500 μM H_2O_2 of Aβ(1-40). The conformational specific antibody A11 that binds to some toxic Aβ oligomers was used in a dot blot essay [57]. There was no evidence that Aβ(1-40)Met^{OX} forms substantial amounts of A11 binding oligomers as no antibody binding was detected. While the OC antibody, which has specificity for fibrilar Aβ, did show some binding to Aβ(1-40)Met^{OX} , see supplemental Figure S2.

3.4 Methionine Oxidation of preformed fibres

Interestingly, although a significant decrease in ThT fluorescence is observed when H_2O_2 is added to monomers, addition of 500 μM H_2O_2 to mature preformed Aβ(1-40) fibres has little effect on total fibre content, as shown in Figure 8a. TEM images of H_2O_2 treated mature fibres incubated for 8 days with 500 μM H_2O_2 show a similar appearance to un-oxidized Aβ(1-40) fibres, Figure 8b, typically exhibiting fibres many microns in length. This indicates that any influence on preformed fibres is a rather slow process in the order of weeks or more.

The amount and morphology of preformed amyloid fibres is largely unaffected by the presence of H_2O_2. This suggests Met^{OX} influences the process of fibre formation, particularly the nucleation and elongation phases, but any effect on stability of fibres once formed must be slow. Although H_2O_2 is a very small molecule, there may be some protection of Met because this residue is buried within the fibres. To further explore these processes seeding experiments were carried out. Here nucleating seeds of unoxidized Aβ(1-40) were added to oxidized Aβ(1-40)Met^{OX} monomer with ThT fluorescence monitored, shown in Figure 9. Even when preformed nucleating seeds are added to oxidized Ab(1-40)Met^{OX} monomer very few fibres are generated, suggesting elongation of fibres does not readily take place once Aβ is oxidized to Met^{OX} even in the presence of seeding preformed fibres.

4. Discussion

Numerous studies have shown evidence of increased levels of oxidative damage in the brain tissue, as well as in the cerebrospinal fluid (CSF) and plasma of patients with AD, for a review see Butterfield *et al* [3]. Indeed, a proportion of Aβ isolated from plaques contains oxidized Methionine (Met^{OX}) [15-17]. Intriguingly, some studies have shown oxidative stress occurs before the appearance of amyloid plaques [4, 5]. The observation that levels of methionine sulphoxide reductase (Msr) activity can influence plaque load has raised the possibility that Aβ oxidation might be a trigger for plaque formation *in vivo* [18, 19]. Previous *in vitro* studies of Met oxidized Aβ(1-40) and Aβ(1-42) have typically indicated a reduction of total

fibre production [32, 33]. Our studies support this assertion but also show that the kinetics of Aβ(1-40) and Aβ(1-42) fibre formation are more complex. Surprisingly although there is a reduction in the total amount of fibres for Aβ(1-40) and a reduced apparent elongation rate, we also show Met oxidation causes reduced lag-time of Aβ(1-40)Met^{OX} fibre formation. The rate of elongation and rate of nucleation are normally thought to be directly correlated [59], but surprisingly, here we show nucleation and elongation rates are inversely correlated when Aβ(1-40) has increasing levels of Met35 oxidation. Although a reduction in fibre load *in vitro* has been described, [32, 33] details of the kinetics of fibre formation have not previously been reported.

Solid State NMR studies have shown Met35 forms close contacts with Val39, Gly38 and Gly37 within the fibre structure of Aβ(1-40) [60, 61]. While a recent structure of Aβ(1-42) fibres indicates Met35 is also closely packed in the core of the fibre [62]. Clearly, generation of Met^{OX} disrupts the hydrophobic packing within fibres. This is supported by molecular dynamics simulations of Aβ(1-40)Met^{OX} self-association [63]. It is likely that Met^{OX} disrupts hydrophobic packing within the fibre and so causes the reduction in the stability of fibres and can explain why the length and total amount of fibres is reduced. Methionine oxidation also reduces the rate of elongation of the fibres, as oxidized Aβ monomer addition to already formed fibres may be less favorable, because the sulphoxide makes the Met sidechain less hydrophobic. This also explains why the Met^{OX} Aβ elongation rates are reduced as self-association with the hydrophobic ends of fibres is less favourable. The reduction in elongation rate is apparent even when monomeric Aβ(1-40)Met^{OX} is seeded with preformed Aβ(1-40) fibres. It is less clear why the lag-times for Aβ(1-40)Met^{OX} is reduced, perhaps it is because fibres with Met^{OX} are less stable and therefore more prone to fragmentation and secondary nucleation, which is supported by our observation that fibres formed with oxidized Aβ(1-40) have a much shorter fragmented appearance. This can dominate fibre growth kinetics [64, 65], as fibre fragmentation will increase the number of ends of fibres available for fibre growth and so reduce lag-times.

Unexpectedly the reverse effect on fibre growth lag-times is observed for Aβ(1-42)Met^{OX}. The differential effects on lag-times between Aβ(1-40)Met^{OX} and Aβ(1-42)Met^{OX} are interesting. There is a good deal of evidence that Aβ(1-40) and Aβ(1-42) follow different pathways to fibre formation and studies suggest that Aβ(1-42) goes through a paranucleus (penta/hexamer) stage that Aβ(1-40) does not [35, 66]. The delay in fibre formation of Aβ(1-42)Met^{OX} may be due to the inhibition of Aβ(1-42) paranuclulus formation when oxidized, perhaps Aβ(1-42)Met^{OX} only forms nucleating oligomers similar to that of Aβ(1-40) and so a longer lag-times are observed. The differential effects of AβMet^{OX} nucleation supports a previous study using chemical cross-linking of Aβ(1-42)Met^{OX} to describe the early nucleating paranucleus [35].

Our simple oxidation method reflects the processes that take place *in vivo* with a mixture of oxidized and un-oxidized Aβ present, with Aβ continuing to be oxidized as increasing amounts of fibres form. At the lower concentrations of H_2O_2 (50 and 100 μM H_2O_2) we have shown Aβ(1-40) fibres will contain a mixture of oxidized and unoxidized Aβ. Interestingly for Aβ(1-40) there is no evidence that the mixed Aβ forms (oxidized and un-oxidized) can "frustrate" nucleation of fibres, as is observed for mixtures of un-oxidized Aβ(1-40) and Aβ(1-42) [67-70].

Met^{OX} is found in the plaques of AD patients [15, 16] but our *in vitro* studies indicate that, at equilibrium, oxidation of Met within Aβ will reduce the total amount of fibres formed. However our studies suggest that partial oxidation of Aβ(1-40) does not initially inhibit formation of fibres and rather than frustrate fibre lag-times, in the case of Aβ(1-40) lag-times are reduced. The oxidation of Met may impact fibre morphology but is unlikely to completely inhibit fibre formation. In addition, Met^{OX} generation in plaques might also occur once fibres are formed as we have shown preformed fibres remain stable (for weeks) in the presence of H_2O_2. *In vivo* the enzyme methionine sulphoxide reductase (Msr) is responsible for the conversion of Met^{OX} back to Met and will reduce surface exposed Met^{OX} [71]. Interestingly, studies of Msr in the brains of AD patients revealed that its activity is reduced compared to control subjects [18]. Indeed, it has recently been shown using an Alzheimer's model in *C. elegans* worms that deletion of the Msr gene modulates Aβ aggregation in the worms [19]. It is unlikely that the Msr enzyme will have access to Met^{OX} buried within the fibres/plaques and so over time Met suphoxide is likely to build up.

5. CONCLUSION

This study demonstrates that Met oxidation of monomeric Aβ(1-40) and Aβ(1-42) generates very different fibre morphologies compared to un-oxidized Aβ; with much shorter (<500 nm) fragmented fibres observed. The reduction in total fibre content for our *in vitro* studies suggests Met^{OX} does not drive fibre formation. Although, once Aβ fibres are formed they remain stable even in the presence of H_2O_2, which suggests Met^{OX} observed in plaques can occur after plaques are formed. The differential effect of Met^{OX} on fibre nucleation for Aβ(1-40) and Aβ(1-42) supports a role for Met35 in directing the structure of nucleating seeds, inhibiting the formation of Aβ(1-42) paranuclei [35]. One mechanism by which H_2O_2 might promote Aβ toxicity is the generation of Aβ fibre fragments and oligomers [65, 72], which are likely to interact with the cellular lipid-bilayer quite differently and methionine oxidation of Aβ is thought to inhibit membrane penetration [73, 74]. Early cell viability studies suggested that Met^{OX} increased the toxicity of Aβ [39]. However, although the anti-oxidant enzyme catalase protect cells from Aβ toxicity [28, 40, 41], more recent studies suggest Aβ with Met^{OX} does not

have enhanced cellular or synapto-toxicity [42-48]. Oxidative processes are certainly a hallmark of AD and anti-oxidant therapeutics might potentially have some efficacy. However clinical trials have yet to indicate that anti-oxidant therapies are beneficial to AD patients [75].

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REFERENCES

[1] M. Prince, J. Jackson, C.P. Ferri, R. Sousa, E. Albanese, W.S. Ribeiro, M. Honyashiki, Alzheimer's disease international world alzheimer report, In International AsD (ed), (2009) pp. 1-96.

[2] J. Hardy, D.J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science, 297 (2002) 353-356.

[3] D.A. Butterfield, T. Reed, S.F. Newman, R. Sultana, Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment, Free Radic Biol Med, 43 (2007) 658-677.

[4] A. Nunomura, G. Perry, G. Aliev, K. Hirai, A. Takeda, E.K. Balraj, P.K. Jones, H. Ghanbari, T. Wataya, S. Shimohama, S. Chiba, C.S. Atwood, R.B. Petersen, M.A. Smith, Oxidative damage is the earliest event in Alzheimer disease, J Neuropathol Exp Neurol, 60 (2001) 759-767.

[5] R.G. Cutler, J. Kelly, K. Storie, W.A. Pedersen, A. Tammara, K. Hatanpaa, J.C. Troncoso, M.P. Mattson, Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease, Proceedings of the National Academy of Sciences, 101 (2004) 2070-2075.

[6] W. Gsell, I. Strein, P. Riederer, The neurochemistry of Alzheimer type, vascular type and mixed type dementias compared, Journal of neural transmission. Supplementum, 47 (1996) 73-101.

[7] R. Pamplona, E. Dalfo, V. Ayala, M.J. Bellmunt, J. Prat, I. Ferrer, M. Portero-Otin, Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets, J Biol Chem, 280 (2005) 21522-21530.

[8] P. Mecocci, U. MacGarvey, M.F. Beal, Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease, Ann Neurol, 36 (1994) 747-751.

[9] T.I. Williams, B.C. Lynn, W.R. Markesbery, M.A. Lovell, Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in Mild Cognitive Impairment and early Alzheimer's disease, Neurobiol Aging, 27 (2006) 1094-1099.

[10] Y.K. Al-Hilaly, T.L. Williams, M. Stewart-Parker, L. Ford, E. Skaria, M. Cole, W.G. Bucher, K.L. Morris, A.A. Sada, J.R. Thorpe, L.C. Serpell, A central role for dityrosine crosslinking of Amyloid-beta in Alzheimer's disease, Acta Neuropathol Commun, 1 (2013) 83.

[11] C.S. Atwood, G. Perry, H. Zeng, Y. Kato, W.D. Jones, K.Q. Ling, X. Huang, R.D. Moir, D. Wang, L.M. Sayre, M.A. Smith, S.G. Chen, A.I. Bush, Copper mediates dityrosine cross-linking of Alzheimer's amyloid-beta, Biochemistry, 43 (2004) 560-568.

[12] R.C. Nadal, S.E. Rigby, J.H. Viles, Amyloid beta-Cu2+ complexes in both monomeric and fibrillar forms do not generate H2O2 catalytically but quench hydroxyl radicals, Biochemistry, 47 (2008) 11653-11664.

[13] C. Schoneich, T.D. Williams, Cu(II)-catalyzed oxidation of beta-amyloid peptide targets His13 and His14 over His6: Detection of 2-Oxo-histidine by HPLC-MS/MS, Chem Res Toxicol, 15 (2002) 717-722.

[14] S.H. Lee, H. Kyung, R. Yokota, T. Goto, T. Oe, Hydroxyl Radical-Mediated Novel Modification of Peptides: N-Terminal Cyclization through the Formation of alpha-Ketoamide, Chem Res Toxicol, 28 (2015) 59-70.

[15] J. Dong, C.S. Atwood, V.E. Anderson, S.L. Siedlak, M.A. Smith, G. Perry, P.R. Carey, Metal binding and oxidation of amyloid-beta within isolated senile plaque cores: Raman microscopic evidence, Biochemistry, 42 (2003) 2768-2773.

[16] J. Naslund, A. Schierhorn, U. Hellman, L. Lannfelt, A.D. Roses, L.O. Tjernberg, J. Silberring, S.E. Gandy, B. Winblad, P. Greengard, et al., Relative abundance of Alzheimer A beta amyloid peptide variants in Alzheimer disease and normal aging, Proc Natl Acad Sci U S A, 91 (1994) 8378-8382.

[17] Y.M. Kuo, T.A. Kokjohn, T.G. Beach, L.I. Sue, D. Brune, J.C. Lopez, W.M. Kalback, D. Abramowski, C. Sturchler-Pierrat, M. Staufenbiel, A.E. Roher, Comparative analysis of amyloid-beta chemical structure and amyloid plaque morphology of transgenic mouse and Alzheimer's disease brains, J Biol Chem, 276 (2001) 12991-12998.

[18] S.P. Gabbita, M.Y. Aksenov, M.A. Lovell, W.R. Markesbery, Decrease in peptide methionine sulfoxide reductase in Alzheimer's disease brain, J Neurochem, 73 (1999) 1660-1666.
[19] A.N. Minniti, M.S. Arrazola, M. Bravo-Zehnder, F. Ramos, N.C. Inestrosa, R. Aldunate, The protein oxidation repair enzyme methionine sulfoxide reductase a modulates Abeta aggregation and toxicity in vivo, Antioxid Redox Signal, 22 (2015) 48-62.
[20] W. Vogt, Oxidation of methionyl residues in proteins: tools, targets, and reversal, Free Radic Biol Med, 18 (1995) 93-105.
[21] B. Halliwell, M.V. Clement, L.H. Long, Hydrogen peroxide in the human body, FEBS Lett, 486 (2000) 10-13.
[22] M.A. Lovell, J.D. Robertson, W.J. Teesdale, J.L. Campbell, W.R. Markesbery, Copper, iron and zinc in Alzheimer's disease senile plaques, J Neurol Sci, 158 (1998) 47-52.
[23] L.M. Miller, Q. Wang, T.P. Telivala, R.J. Smith, A. Lanzirotti, J. Miklossy, Synchrotron-based infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with beta-amyloid deposits in Alzheimer's disease, J Struct Biol, 155 (2006) 30-37.
[24] C.J. Sarell, C.D. Syme, S.E. Rigby, J.H. Viles, Copper(II) binding to amyloid-beta fibrils of Alzheimer's disease reveals a picomolar affinity: stoichiometry and coordination geometry are independent of Abeta oligomeric form, Biochemistry, 48 (2009) 4388-4402.
[25] T. Rival, R.M. Page, D.S. Chandraratna, T.J. Sendall, E. Ryder, B. Liu, H. Lewis, T. Rosahl, R. Hider, L.M. Camargo, M.S. Shearman, D.C. Crowther, D.A. Lomas, Fenton chemistry and oxidative stress mediate the toxicity of the beta-amyloid peptide in a Drosophila model of Alzheimer's disease, Eur J Neurosci, 29 (2009) 1335-1347.
[26] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 4th ed. ed., Oxford University Press, Place Published, 2007.
[27] K.J. Barnham, F. Haeffner, G.D. Ciccotosto, C.C. Curtain, D. Tew, C. Mavros, K. Beyreuther, D. Carrington, C.L. Masters, R.A. Cherny, R. Cappai, A.I. Bush, Tyrosine gated electron transfer is key to the toxic mechanism of Alzheimer's disease beta-amyloid, Faseb Journal, 18 (2004) 1427-1429.
[28] C. Opazo, X. Huang, R.A. Cherny, R.D. Moir, A.E. Roher, A.R. White, R. Cappai, C.L. Masters, R.E. Tanzi, N.C. Inestrosa, A.I. Bush, Metalloenzyme-like activity of Alzheimer's disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H(2)O(2), J Biol Chem, 277 (2002) 40302-40308.
[29] C. Hureau, P. Faller, Abeta-mediated ROS production by Cu ions: structural insights, mechanisms and relevance to Alzheimer's disease, Biochimie, 91 (2009) 1212-1217.
[30] L.E. Cassagnes, V. Herve, F. Nepveu, C. Hureau, P. Faller, F. Collin, The catalytically active copper-amyloid-Beta state: coordination site responsible for reactive oxygen species production, Angew Chem Int Ed Engl, 52 (2013) 11110-11113.
[31] G. Meloni, V. Sonois, T. Delaine, L. Guilloreau, A. Gillet, J. Teissie, P. Faller, M. Vasak, Metal swap between Zn7-metallothionein-3 and amyloid-beta-Cu protects against amyloid-beta toxicity, Nat Chem Biol, 4 (2008) 366-372.
[32] L. Hou, I. Kang, R.E. Marchant, M.G. Zagorski, Methionine 35 oxidation reduces fibril assembly of the amyloid abeta-(1-42) peptide of Alzheimer's disease, J Biol Chem, 277 (2002) 40173-40176.
[33] A.A. Watson, D.P. Fairlie, D.J. Craik, Solution structure of methionine-oxidized amyloid beta-peptide (1-40). Does oxidation affect conformational switching?, Biochemistry, 37 (1998) 12700-12706.
[34] L. Hou, H. Shao, Y. Zhang, H. Li, N.K. Menon, E.B. Neuhaus, J.M. Brewer, I.J. Byeon, D.G. Ray, M.P. Vitek, T. Iwashita, R.A. Makula, A.B. Przybyla, M.G. Zagorski, Solution NMR studies of the A beta(1-40) and A beta(1-42) peptides establish that the Met35 oxidation state affects the mechanism of amyloid formation, J Am Chem Soc, 126 (2004) 1992-2005.
[35] G. Bitan, B. Tarus, S.S. Vollers, H.A. Lashuel, M.M. Condron, J.E. Straub, D.B. Teplow, A molecular switch in amyloid assembly: Met35 and amyloid beta-protein oligomerization, J Am Chem Soc, 125

(2003) 15359-15365.
[36] M. Palmblad, A. Westlind-Danielsson, J. Bergquist, Oxidation of methionine 35 attenuates formation of amyloid beta -peptide 1-40 oligomers, J Biol Chem, 277 (2002) 19506-19510.
[37] S.W. Snyder, U.S. Ladror, W.S. Wade, G.T. Wang, L.W. Barrett, E.D. Matayoshi, H.J. Huffaker, G.A. Krafft, T.F. Holzman, Amyloid-beta aggregation: selective inhibition of aggregation in mixtures of amyloid with different chain lengths, Biophysical Journal, 67 (1994) 1216-1228.
[38] L. Hou, H.G. Lee, F. Han, J.M. Tedesco, G. Perry, M.A. Smith, M.G. Zagorski, Modification of amyloid-beta1-42 fibril structure by methionine-35 oxidation, J Alzheimers Dis, 37 (2013) 9-18.
[39] B. Seilheimer, B. Bohrmann, L. Bondolfi, F. Muller, D. Stuber, H. Dobeli, The toxicity of the Alzheimer's beta-amyloid peptide correlates with a distinct fiber morphology, J Struct Biol, 119 (1997) 59-71.
[40] C. Behl, J.B. Davis, R. Lesley, D. Schubert, Hydrogen peroxide mediates amyloid beta protein toxicity, Cell, 77 (1994) 817-827.
[41] X. Huang, C.S. Atwood, M.A. Hartshorn, G. Multhaup, L.E. Goldstein, R.C. Scarpa, M.P. Cuajungco, D.N. Gray, J. Lim, R.D. Moir, R.E. Tanzi, A.I. Bush, The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction, Biochemistry, 38 (1999) 7609-7616.
[42] F. Misiti, M.E. Clementi, G. Tringali, M. Vairano, F. Orsini, M. Pezzotti, P. Navarra, B. Giardina, G. Pozzoli, Fragment 31-35 of beta-amyloid peptide induces neurodegeneration in rat cerebellar granule cells via bax gene expression and caspase-3 activation - A crucial role for the redox state of methionine-35 residue, Neurochemistry International, 49 (2006) 525-532.
[43] M.E. Clementi, F. Misiti, Substitution of methionine 35 inhibits apoptotic effects of A beta(31-35) and A beta(25-35) fragments of amyloid-beta protein in PC12 cells, Med Sci Monitor, 11 (2005) Br381-Br385.
[44] M.E. Clementi, S. Marini, M. Coletta, F. Orsini, B. Giardina, F. Misiti, A beta(31-35) and A beta(25-35) fragments of amyloid beta-protein induce cellular death through apoptotic signals: Role of the redox state of methionine-35, Febs Letters, 579 (2005) 2913-2918.
[45] F. Misiti, B. Sampaolese, M. Pezzotti, S. Marini, M. Coletta, L. Ceccarelli, B. Giardina, M.E. Clementi, A beta(31-35) peptide induce apoptosis in PC 12 cells: Contrast with A beta(25-35) peptide and examination of underlying mechanisms, Neurochemistry International, 46 (2005) 575-583.
[46] F. Misiti, M.E. Clementi, B. Giardina, Oxidation of methionine 35 reduces toxicity of the amyloid beta-peptide(1-42) in neuroblastoma cells (IMR-32) via enzyme methionine sulfoxide reductase A expression and function, Neurochemistry International, 56 (2010) 597-602.
[47] R. Piacentini, C. Ripoli, L. Leone, F. Misiti, M.E. Clementi, M. D'Ascenzo, B. Giardina, G.B. Azzena, C. Grassi, Role of methionine 35 in the intracellular Ca(2+) homeostasis dysregulation and Ca(2+)-dependent apoptosis induced by amyloid beta-peptide in human neuroblastoma IMR32 cells, J Neurochem, 107 (2008) 1070-1082.
[48] C. Ripoli, R. Piacentini, E. Riccardi, L. Leone, D.D.L. Puma, G. Bitan, C. Grassi, Effects of different amyloid beta-protein analogues on synaptic function, Neurobiol Aging, 34 (2013) 1032-1044.
[49] M. Friedemann, E. Helk, A. Tiiman, K. Zovo, P. Palumaa, V. Tougu, Oxidation of Methionine-35 in Alzheimer's amyloid-b peptide and the aggregation of the oxidized peptide, Biochemistry and Biophysics Reports, 3 (2015) 94-99.
[50] Y. Fezoui, D.M. Hartley, J.D. Harper, R. Khurana, D.M. Walsh, M.M. Condron, D.J. Selkoe, P.T. Lansbury, Jr., A.L. Fink, D.B. Teplow, An improved method of preparing the amyloid beta-protein for fibrillogenesis and neurotoxicity experiments, Amyloid, 7 (2000) 166-178.
[51] D.B. Teplow, Preparation of amyloid beta-protein for structural and functional studies, Method Enzymol, 413 (2006) 20-33.
[52] N.D. Younan, J.H. Viles, A Comparison of Three Fluorophores for the Detection of Amyloid Fibers and Prefibrillar Oligomeric Assemblies. ThT (Thioflavin T); ANS (1-Anilinonaphthalene-8-sulfonic Acid);

and bisANS (4,4 '-Dianilino-1,1 '-binaphthyl-5,5 '-disulfonic Acid), Biochemistry, 54 (2015) 4297-4306.
[53] G. Meisl, J.B. Kirkegaard, P. Arosio, T.C. Michaels, M. Vendruscolo, C.M. Dobson, S. Linse, T.P. Knowles, Molecular mechanisms of protein aggregation from global fitting of kinetic models, Nat Protoc, 11 (2016) 252-272.
[54] M. Biancalana, S. Koide, Molecular mechanism of Thioflavin-T binding to amyloid fibrils, Biochim Biophys Acta, 1804 (2010) 1405-1412.
[55] V.N. Uversky, J. Li, A.L. Fink, Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure, J. Biol. Chem., 276 (2001) 44284-44296.
[56] N.D. Younan, C.J. Sarell, P. Davies, D.R. Brown, J.H. Viles, The cellular prion protein traps Alzheimer's Abeta in an oligomeric form and disassembles amyloid fibers, Faseb J, 27 (2013) 1847-1858.
[57] R. Kayed, E. Head, J.L. Thompson, T.M. McIntire, S.C. Milton, C.W. Cotman, C.G. Glabe, Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis, Science, 300 (2003) 486-489.
[58] C. Schoneich, Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease, Biochim Biophys Acta, 1703 (2005) 111-119.
[59] M. Fandrich, Absolute correlation between lag time and growth rate in the spontaneous formation of several amyloid-like aggregates and fibrils, Journal of molecular biology, 365 (2007) 1266-1270.
[60] A.T. Petkova, W.M. Yau, R. Tycko, Experimental constraints on quaternary structure in Alzheimer's beta-amyloid fibrils, Biochemistry, 45 (2006) 498-512.
[61] I. Bertini, L. Gonnelli, C. Luchinat, J. Mao, A. Nesi, A new structural model of Abeta40 fibrils, J Am Chem Soc, 133 (2011) 16013-16022.
[62] M. Schmidt, A. Rohou, K. Lasker, J.K. Yadav, C. Schiene-Fischer, M. Fandrich, N. Grigorieff, Peptide dimer structure in an Abeta(1-42) fibril visualized with cryo-EM, Proc Natl Acad Sci U S A, 112 (2015) 11858-11863.
[63] A.M. Brown, J.A. Lemkul, N. Schaum, D.R. Bevan, Simulations of monomeric amyloid beta-peptide (1-40) with varying solution conditions and oxidation state of Met35: implications for aggregation, Arch Biochem Biophys, 545 (2014) 44-52.
[64] T.P. Knowles, C.A. Waudby, G.L. Devlin, S.I. Cohen, A. Aguzzi, M. Vendruscolo, E.M. Terentjev, M.E. Welland, C.M. Dobson, An analytical solution to the kinetics of breakable filament assembly, Science, 326 (2009) 1533-1537.
[65] W.F. Xue, A.L. Hellewell, W.S. Gosal, S.W. Homans, E.W. Hewitt, S.E. Radford, Fibril fragmentation enhances amyloid cytotoxicity, J Biol Chem, 284 (2009) 34272-34282.
[66] Y.R. Chen, C.G. Glabe, Distinct early folding and aggregation properties of Alzheimer amyloid-beta peptides Abeta40 and Abeta42: stable trimer or tetramer formation by Abeta42, J Biol Chem, 281 (2006) 24414-24422.
[67] D. Scheuner, C. Eckman, M. Jensen, X. Song, M. Citron, N. Suzuki, T.D. Bird, J. Hardy, M. Hutton, Kukull, W., E. Larson, E. Levy-Lahad, M. Viitanen, E. Peskind, P. Poorkaj, G. Schellenberg, Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease, Nat. Med., 2 (1996) 864-870.
[68] K. Duff, C. Eckman, C. Zehr, X. Yu, C.M. Prada, J. Perez-tur, M. Hutton, L. Buee, Y. Harigaya, D. Yager, D. Morgan, M.N. Gordon, L. Holcomb, L. Refolo, B. Zenk, J. Hardy, S. Younkin, Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1, Nature, 383 (1996) 710-713.
[69] M. Citron, D. Westaway, W. Xia, G. Carlson, T. Diehl, G. Levesque, K. Johnson-Wood, M. Lee, P. Seubert, A. Davis, D. Kholodenko, R. Motter, R. Sherrington, B. Perry, H. Yao, R. Strome, I. Lieberburg, J. Rommens, S. Kim, D. Schenk, P. Fraser, P. St George Hyslop, D.J. Selkoe, Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and

transgenic mice, Nat. Med., 3 (1997) 67-72.
[70] Y. Xiao, B. Ma, D. McElheny, S. Parthasarathy, F. Long, M. Hoshi, R. Nussinov, Y. Ishii, Abeta(1-42) fibril structure illuminates self-recognition and replication of amyloid in Alzheimer's disease, Nat Struct Mol Biol, 22 (2015) 499-505.
[71] R.L. Levine, L. Mosoni, B.S. Berlett, E.R. Stadtman, Methionine residues as endogenous antioxidants in proteins, Proc Natl Acad Sci U S A, 93 (1996) 15036-15040.
[72] B.A. Chromy, R.J. Nowak, M.P. Lambert, K.L. Viola, L. Chang, P.T. Velasco, B.W. Jones, S.J. Fernandez, P.N. Lacor, P. Horowitz, C.E. Finch, G.A. Krafft, W.L. Klein, Self-assembly of Abeta(1-42) into globular neurotoxins, Biochemistry, 42 (2003) 12749-12760.
[73] K.J. Barnham, G.D. Ciccotosto, A.K. Tickler, F.E. Ali, D.G. Smith, N.A. Williamson, Y.H. Lam, D. Carrington, D. Tew, G. Kocak, I. Volitakis, F. Separovic, C.J. Barrow, J.D. Wade, C.L. Masters, R.A. Cherny, C.C. Curtain, A.I. Bush, R. Cappai, Methionine oxidation and the toxic principle of the beta-amyloid peptide from Alzheimer's disease, Journal of Neurochemistry, 87 (2003) 99-99.
[74] K.J. Barnham, G.D. Ciccotosto, A.K. Tickler, F.E. Ali, D.G. Smith, N.A. Williamson, Y.H. Lam, D. Carrington, D. Tew, G. Kocak, I. Volitakis, F. Separovic, C.J. Barrow, J.D. Wade, C.L. Masters, R.A. Cherny, C.C. Curtain, A.I. Bush, R. Cappai, Neurotoxic, redox-competent Alzheimer's beta-amyloid is released from lipid membrane by methionine oxidation, Journal of Biological Chemistry, 278 (2003) 42959-42965.
[75] Y. Gilgun-Sherki, E. Melamed, D. Offen, Antioxidant treatment in Alzheimer's disease: current state, J Mol Neurosci, 21 (2003) 1-11.

Table 1: Methionine oxidation rates with H_2O_2

H_2O_2	100 μM	200 μM	500 μM	5000 μM
50% Met^{OX}	5 days	24 hrs	5 hrs	1 hr

50% Met^{OX} is the time for half the Met35 to be oxidized in 10 μM monomeric Aβ(1-40). As detected by ^1H NMR at pH 7.4, 30 ^oC.

Figure Captions:

Figure 1: ^{1}H NMR in aliphatic region to monitor Aβ(1-40) Methionine oxidation. a) Structure of Methionine sulphoxide b) ^{1}H NMR spectra of 10 μM of monomeric Aβ(1-40) incubated with 500 μM H_2O_2 recorded every hour, pH 7.4; 10 mM phosphate buffer. ϵCH_3 of Met at 2.1 ppm, ϵCH_3 of Met-sulphoxide at 2.7 ppm. c) Peak intensity of Met ϵCH_3 (o) and Met-sulphoxide ϵCH_3 (•) over time, for 500 μM H_2O_2 incubation. Met35 is oxidized within 20 hours.

Figure 2: Electrospray Mass Spectra of Aβ(1–40). a) Aβ(1–40) alone; b) Aβ(1–40) incubated with 500 μM H_2O_2 for 7 hours. Showing Aβ(1–40) with +4 charge, Aβ(1–40) oxidation shows a 16 Da increase in mass.

Figure 3: Fibre growth kinetics of Aβ(1-40) in the presence of H_2O_2. Aβ(1-40) 10 μM, incubated with no H_2O_2 (Black traces) and 300 μM H_2O_2 (Red traces), 4 or 5 traces for each condition. ThT indicates H_2O_2 accelerates the nucleation of Aβ(1-40) fibre formation. Fibres were grown in 100 mM HEPES and 160 mM NaCl at pH 7.4 at 30 °C with mild agitation. *AFU*, arbitrary fluorescence units, from 20 μM ThT binding to fibres. significance. Bar-graphs compare mean kinetic parameters k_{app}, t_{lag} and total fibre fluorescence at equilibrium in the presence and absence of 300 μM H_2O_2 * indicates a $P < 0.05$.

Figure 4: Aβ(1– 40) fibril growth in the presence of H_2O_2. Fibrillisation of 10 μM Aβ(1– 40) was monitored using ThT fluorescence in the absence and presence of 50, 100, 200, 300 and 500 μM of H_2O_2. The median trace of six or more individual traces for each condition is shown (All traces are shown in a supplemental figure S1). Fibres were grown in 100 mM HEPES and 160 mM NaCl at pH 7.4 and 30 ^oC with different amounts of agitation: (Row-a) 60 s of agitation and (Row-b) 45 s of agitation every 30 min. Bar-graphs compare mean kinetic parameters k_{app}, t_{lag} and total fibre fluorescence at equilibrium, in the absence and presence of the range of H_2O_2 concentrations. * indicates a $P < 0.05$. (Δ would not fit sigmodal growth curve)

Figure 5: Fibre growth kinetics of Aβ(1-42) incubated with H_2O_2. 10 μM Aβ(1-42) with 500 μM H_2O_2 (a); and 1000 μM H_2O_2 (b). All samples pH 7.4, 160 mM NaCl, 100 mM HEPES buffer and 20 μM ThT. Four traces are shown for each condition. H_2O_2 oxidation inhibits Aβ(1-42) fibre formation.

Figure 6: TEM images of Aβ(1– 40) assemblies. Column: a) No H_2O_2; b) 100 μM H_2O_2 ; c) 300 μM H_2O_2 ; d) 500 μM H_2O_2. Top-row; scale-bar 5 μm and bottom-row; scale-bar 100 nm. Increasing concentrations of H_2O_2 reduces the number and length of Aβ(1-40) fibres. Aβ(1-40) incubated for 400 hrs with mild agitation, 100 mM HEPES and 160 mM NaCl at pH 7.4 and 30 °C.

Figure 7: TEM images of Aβ(1– 42) assemblies. a) No H_2O_2; b) 1000 μM H_2O_2. H_2O_2 oxidation reduces the number and length of Aβ(1-42) fibres. Aβ(1-42) incubated for 240 hrs with mild agitation, 100 mM HEPES and 160 mM NaCl at pH 7.4 and 30 °C.

Figure 8: **Addition of H_2O_2 to preformed fibres.** a) traces are ThT signal after adding 500 μM H_2O_2 to preformed Aβ(1-40) fibre (10 μM) after 190 hours, b) is 10 μM Aβ(1-40) control, no H_2O_2 added. c) and d) shows TEM image of mature fibre with 500 μM H_2O_2 added to preformed fibres and then further incubated for 5 days. Addition of H_2O_2 to preformed fibres has little effect on fibre content or morphology. Fibres were generated by incubation of Aβ(1-40) at 10 μM, 37 °C in 100 mM HEPES buffer at pH 7.4 with 160 mM NaCl and 20 μM ThT.

Figure 9: Seeded fibre grow of Aβ(1-40)Met^{ox}. Monitored by ThT fluorescence a) Aβ(1-40) alone; b) Aβ(1-40) with 1000 μM H_2O_2; c) Aβ(1-40) seed with 20% un-oxidized mature fibres, no H_2O_2; d) Aβ(1-40) with 1000 μM H_2O_2 seed with 20% un-oxidized mature fibres. Aβ(1-40) 10 μM, 100 mM HEPES buffer at pH 7.4 with 160 mM NaCl and 20 μM ThT. H_2O_2 inhibits total fibre content even when seeded with nucleating fibres.