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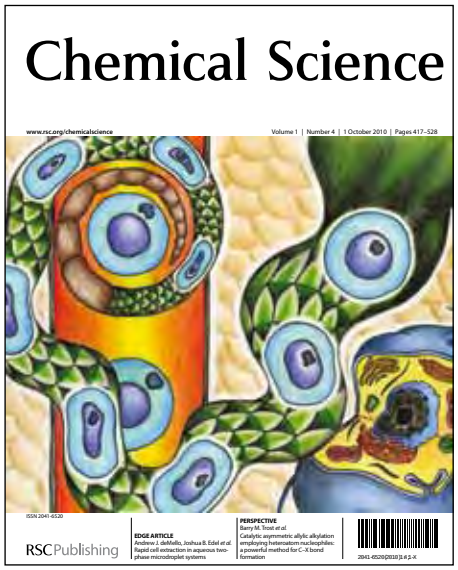

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EDGE ARTICLE

Synthetic Dityrosine-linked β-Amyloid Dimers Form Stable, Soluble, Neurotoxic Oligomers

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Substantial evidence suggests that soluble oligomers of Aβ are the neurotoxic form resulting in progression of Alzheimer's disease (AD). Tyrosine-10 has been identified as a pivotal residue in the neurotoxicity of Aβ and dityrosine cross-linked Aβ dimers have been proposed as the physiologically relevant Aβ species linked to the progression of AD. We describe the synthesis and characterization of dityrosine-linked Aβ dimers and demonstrate that, in contrast to other covalently-linked Aβ dimers, dityrosine-linked Aβ dimers form discrete, stable, soluble aggregates. Furthermore, dityrosine-linked Aβ dimers display increased toxicity in a neuronal cell-line assay compared with the corresponding monomer, consistent with the hypothesis that dityrosine-linked Aβ dimers are implicated in the progression of AD.

Introduction

Alzheimer's disease (AD) is the most common age related neurodegenerative disease, and is characterised by the deposition of extracellular amyloid plaques composed predominantly of β-amyloid (Aβ) peptides. Though the association of Aβ with AD is well established, extensive evidence now suggests that soluble oligomers of Aβ, particularly 'low-*n*' oligomers such as dimers or trimers, are the neurotoxic form of Aβ, rather than amyloid plaque deposits.[1-6] Soluble Aβ dimers extracted directly from the cerebral cortex of subjects with AD have been shown to disrupt the memory of a learned behaviour in normal rats.[7] Blood levels of Aβ dimers have also been shown to be significantly raised in AD patients compared with healthy controls.[8] Further, it has been shown that insoluble amyloid plaque cores from the AD brain cortex do not inhibit long-term potentiation (LTP) unless they are solubilised to release Aβ dimers,[7] further suggesting Aβ dimers are synaptotoxic.

Synthetic Aβ dimers have been shown to display different biological and biophysical properties than the corresponding monomers. Selkoe and co-workers have prepared disulfide-linked, modified Aβ dimer **1** (Figure 1) and showed that this peptide dimer inhibits LTP in mice, whereas the monomer does not.[7] We have shown that alkyl-linked Aβ dimer **2** fibrilises at a greater rate than the corresponding monomer.[9] However, these synthetic alkyl- and disulfide-linked Aβ dimers clearly do not represent the physiologically relevant Aβ dimers in AD.

Figure 1. Synthetic covalently-linked Aβ dimers.

Tyrosine has been identified as an important amino acid for both the conformation and biological activity of Aβ.[10,11] Tyrosine-10 has been proposed as a pivotal residue in the formation of Aβ dimers through the formation of a dityrosine linkage, with variants of Aβ lacking tyrosine at position 10 being non-toxic.[12] Further, evidence suggests dityrosine[13] and dityrosine-linked Aβ42 dimer levels[8] are elevated in AD patient brains and blood, respectively. Incubation of Aβ under oxidative conditions induces both dityrosine formation and the SDS-resistant oligomerization of Aβ, the latter feature being characteristic of neurotoxic, soluble Aβ extracted from the AD brain.[14,15] Substantial evidence therefore points to dityrosine-linked Aβ dimers being one of the neurotoxic species in AD. However, studies of the properties of dityrosine-linked Aβ are limited due to the absence of a method to prepare such peptides as discrete species. Short dityrosine-linked Aβ dimers have been prepared by oxidation with peroxidase or Cu^{2+}/H_2O_2,[16-18] but the method is inefficient with Aβ peptides longer than Aβ16.

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Palladium-catalysed cross-linking of iodotyrosine-containing Aβ fragments is similarly limited to production of short peptide dimers.[19]

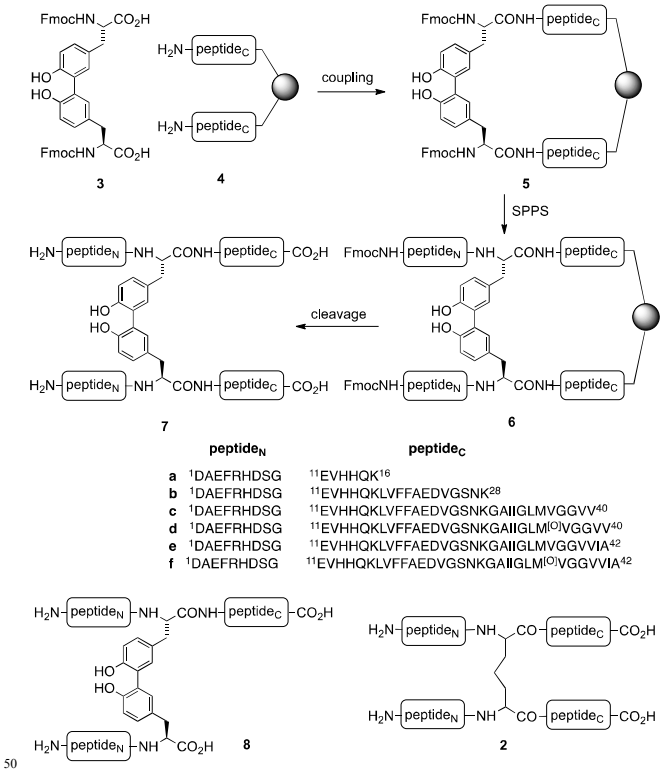
We herein disclose the first chemical synthesis of physiologically-relevant, dityrosine cross-linked Aβ dimers. Our studies show that the nature of the cross-link has a dramatic effect on the properties of the corresponding Aβ dimers. Specifically, dityrosine-linked Aβ dimers are slow to fibrilise, forming *long-lived, soluble oligomeric aggregates*, and are significantly more neurotoxic than the corresponding monomeric peptides.

Results and Discussion

Synthesis of dityrosine-linked Aβ dimers

Synthesis of dityrosine-linked Aβ dimers **7a**–**f** proceeded through modification and optimization of our method developed for the preparation of diaminopimelate- (DAP)-linked peptide dimers,[9] wherein an Fmoc-protected bis amino acid (such as **3**) was coupled to two contiguous resin-bound peptides **4** to give bis-ligated adduct **5**. Peptide extension through standard Fmoc-based SPPS to give **6** was followed by cleavage from the resin to give the peptide dimer **7** (Scheme 1). Modification of the protocol was necessary due to the presence of unprotected side-chain phenolic groups in Fmoc-dityrosine **3**. Accordingly, coupling of Fmoc-dityrosine **3**[20,21] was performed under non-basic conditions, employing a mixture of DIC and HOBt in place of the standard HBTU/DIPEA coupling conditions. Optimization of the dityrosine coupling step was investigated through synthesis of the Aβ16 dimer **7a**, by varying the ratio of Fmoc-dityrosine **3** and coupling agent. Coupling of 1 equiv of dityrosine **3** to resin-bound peptide **4a** gave the corresponding mono-coupled adduct **8a** as the major product through ligation of a single acid group of **3** to the resin. Reducing the amount of dityrosine **3** to 0.5 equiv (1 equiv acid per peptide *N*-terminal amine) similarly yielded the mono-coupled adduct **8a** as the major product, with some bis-adduct **7a**. The use of 0.25 equiv of dityrosine **3** yielded the bis-coupled adduct **7a** (along with unreacted Aβ(11–16) peptide), without the generation of the corresponding mono-coupled adduct **8a**. Subsequent coupling of a further 0.25 equiv of dityrosine **3** increased the amount of bis-coupled adduct **7a**. Accordingly, optimised conditions involved 2–3 sequential couplings of 0.25 equiv of Fmoc-dityrosine **3**. Purification was performed under standard conditions by preparative scale RP-HPLC to give the Aβ16 dityrosine-linked dimer **7a** in >95% purity (see SI).

Preparation of the Aβ28 dityrosine-linked dimer **7b** proceeded according to method for the Aβ16 dimer **7a**, except that a two-step purification protocol was required: RP-HPLC followed by size exclusion chromatography afforded the Aβ28 dimer **7b** in >95% purity (see SI).



Scheme 1. SPPS of dityrosine-linked peptide dimers.

Preparation of the Aβ40 dimer **7c** was achieved following the same method developed for the syntheses of the shorter Aβ peptide dimers. Mass spectrometric analysis of the crude peptide revealed the appearance of the expected molecular ion $[M+6H]^{6+}$ at m/z 1443.9, and an additional +6-charged ion at 1446.4, corresponding to a byproduct 16 amu greater than the dimer **7c**. The [M+16] species presumably arises from oxidation of the methionine residue at position 35 to the methionine sulfoxide ($Met^{[O]}$).[24,25] Treatment of the crude peptide with TMS-Br and thioanisole for 15 minutes resulted in reduction of the oxidised peptide **7cd** to give clean **7c**.[22] An optimised procedure was developed in which addition of TMS-Br and thioanisole to the cleavage mixture resulted in successful reduction of the peptide, thus enabling preparation of the Aβ40 peptide dimer **7c** free from oxidised version **7cd** with no additional steps. Purification of the Aβ40 dimer **7c** was achieved through a two-step RP-HPLC protocol, providing the peptide dimer **7c** in >95% purity (Figure 2). Alternatively, direct incorporation of a $Met^{[O]}$ residue into the peptide sequence gave the fully-oxidised Aβ40-$Met^{[O]}$35 dimer **7d** via the same protocol.

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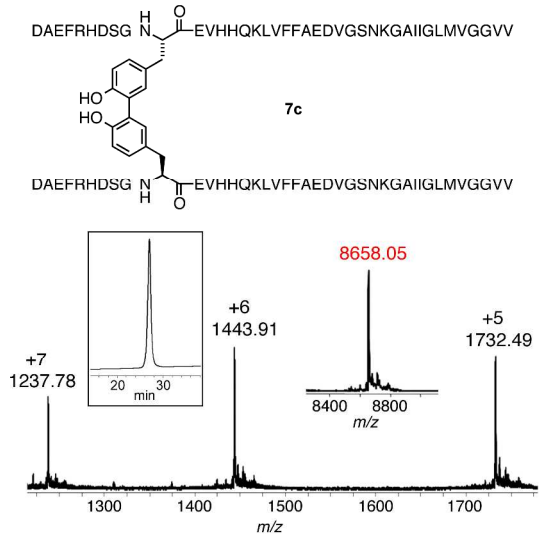

Figure 2. ESI-MS of Aβ40 dityrosine dimer **7c** with deconvoluted MS and HPLC (inset) (calcd. *m/z* 8658.1).

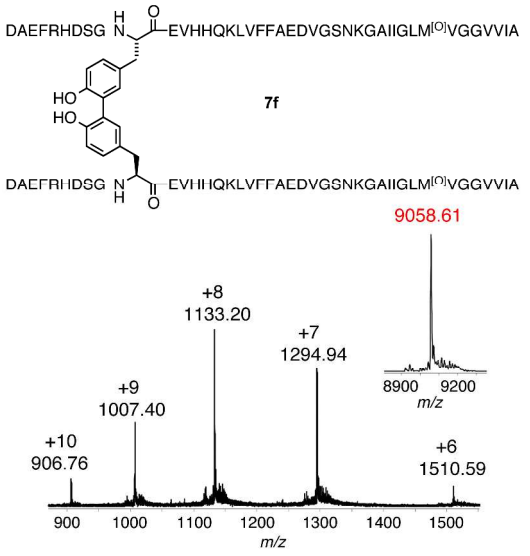

Figure 3. ESI-MS and deconvoluted MS (inset) of Aβ42 dityrosine-linked dimer **7f** (calcd. *m/z* 9058.2).

With the successful preparation of the Aβ40 dimers **7c** and **7d**, the synthesis of the full-length Aβ42 dimer was next investigated. Aβ42 is known to display significantly greater propensity to aggregate than Aβ40.[26] Coupling of Fmoc-dityrosine **3** to the Aβ(11–42) resin-bound peptide **4e** was not successful; only unreacted Aβ(11–42) peptide was detected by mass spectrometric analysis. Further investigations with alternative coupling agents (e.g., HATU, triphosgene) were also unsuccessful. Presumably, on-resin peptide aggregation occludes the *N*-termini of contiguous peptide chains such that coupling to both acid groups of Fmoc-dityrosine **3** is prevented.[27]

The presence of Met$^{[O]}$35 in Aβ peptides has been shown to stabilise the random structure and decrease the rate of β-sheet production due to the increased polarity of the Met$^{[O]}$ side-chain.[28] Thus the incorporation of an oxidised methionine was anticipated to result in a more soluble peptide with less hydrophobicity-induced aggregation. Indeed, coupling of Fmoc-dityrosine **3** to resin-bound Aβ(11–42)Met$^{[O]}$35 peptide **4f** was found to be successful, where coupling to Aβ(11–42) peptide **4e** was not. Mass spectrometric analysis of the cleavage mixture revealed the presence of both the mono-coupled product **8f** and the desired Aβ42-Met$^{[O]}$35 dityrosine-linked dimer **7f** (see SI). However, the Aβ42 dityrosine-linked dimer **7f** was extremely prone to aggregation resulting in purification of this peptide being much more difficult than for the Aβ40 dimers **7c** and **7d**, with standard conditions failing to separate the Aβ42-Met$^{[O]}$35 dimer **7f** from the mono-coupled product **8f**. After considerable experimentation, purification of the oxidised Aβ42 dimer **7f** was achieved by size-exclusion chromatography eluting with 70% formic acid in water. Formic acid has previously been used to prevent aggregation of Aβ peptide solutions.[29,30] However, formation of formate esters of Aβ occurred if the peptide was left in the eluting solvent for >30 min.[31] Accordingly, immediate removal of the formic acid by elution through a hydrophilic–lipophilic balance (HLB) cartridge was necessary, furnishing small amounts of the dimer **7f** (Figure 3).

Particle Size Analysis by Dynamic Light Scattering

We have previously shown that alkyl-linked Aβ dimers exhibit formation of large, polydisperse aggregates soon after dissolution, similar to the aggregation of the corresponding Aβ monomers.[9] The aggregation properties of the dityrosine-linked Aβ dimers **7a–f** were investigated using dynamic light scattering (DLS).

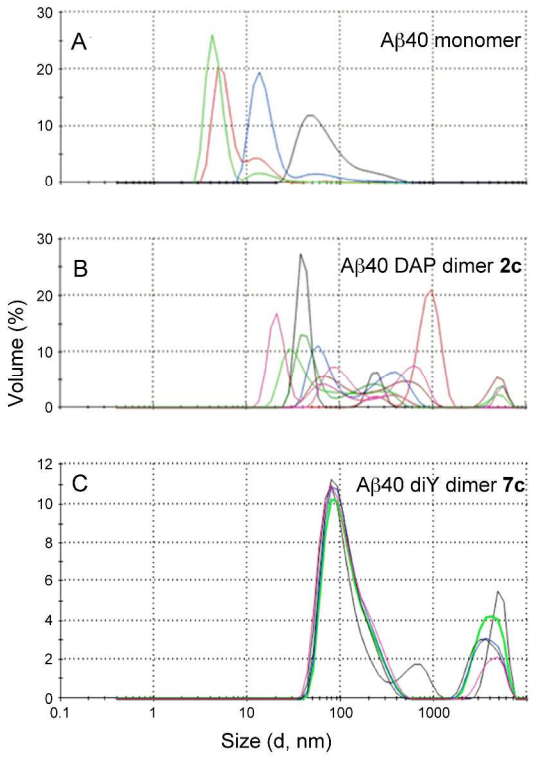

Figure 4. Particle size analysis by DLS: repeat measurements shown for (A) Aβ40 monomer; (B) Aβ40 DAP dimer **2c**; (C) Aβ40 dityrosine dimer **7c**.

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The dityrosine-linked dimers **7a** and **7b** display a propensity towards the formation of large, soluble aggregates (see SI), in stark contrast to the corresponding monomer and alkyl-linked dimer **2a**, which both display size distribution profiles consistent with monomeric states at the same concentration.[9]

The dityrosine-linked Aβ40 dimer **7c** was also observed to reproducibly form high-order aggregates, with the majority of particles of *ca.* 80 nm diameter (Figure 4C). The narrow dispersity of the dityrosine-linked Aβ40 dimer **7c** aggregates contrasts with both the Aβ40 monomer and the corresponding DAP-linked dimer **2c**, which display a high polydispersity in formation of aggregates (Figure 4A/B).[9] The Aβ42-Met$^{[O]}$35 dimer **7f** was found to be so prone to aggregation that consistent preparations of solutions of this peptide were difficult to reproduce. Accordingly, the Aβ42-Met$^{[O]}$35 dimer **7f** was not subject to these or subsequent assays. Nevertheless, the DLS studies show that the presence of a dityrosine cross-link in Aβ peptide dimers specifically promotes the formation of high-order, soluble, oligomeric forms of Aβ.

Fibrilization Kinetics using ThT Assay

The effect of the dityrosine cross-link on the formation of amyloid fibrils was next investigated. We have previously shown that DAP-linked Aβ40 dimer undergoes fibrilization with a considerably reduced lag-time compared with the Aβ40 monomer, consistent with the dimer representing the first stage of nucleation toward the fibrilization of the peptide. Intriguingly, the dityrosine-linked Aβ40 dimer **7c** exhibited a greatly *decelerated* amyloid formation process, with no fibrilization detected after two days by ThT assay (Figure 5: diY-dimer **7c**, blue; DAP-dimer **2c**, red; monomer, black). The combination of DLS and ThT studies show that whilst the dityrosine-linked dimers form high order aggregates, these do not transform readily to amyloid fibrils; i.e. they form *long-lived, soluble aggregates*.

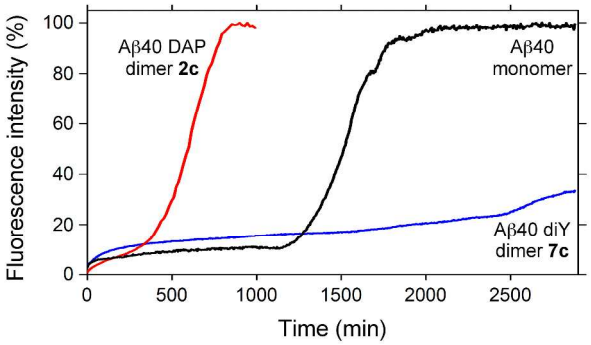

Figure 5. ThT assay of fibril formation of Aβ40 monomer (black), Aβ40 DAP-linked dimer **2c** (red) and Aβ40 dityrosine-linked dimer **7c** (blue); Relative fluorescence intensity; excitation 444 nm, emission 480 nm.

Electron Microscopy

In order to corroborate the DLS and ThT assay results, the Aβ monomer and dimers were aged over 7 days and analysed by electron microscopy. The microscopy results are in close accord with the ThT assays, with the DAP-linked Aβ40 dimer **2c** showing extensive fibril formation after one day, while the Aβ40 monomer requires 2–3 days for extensive fibril formation.

In contrast, the dityrosine-linked Aβ40 dimer **7c** shows dramatically reduced fibril formation (Figure 6). After one day, no fibrils are seen; a high concentration of globular aggregates is observed. After 2–3 days, few fibrils are observed, with the globular aggregates predominating. Only after 7 days are significant numbers of fibrils observed, though substantial quantities of globular aggregates remain. The combination of DLS, ThT and EM findings are all consistent with the dityrosine-linked Aβ dimers forming high-order aggregates that do not transform readily to amyloid fibrils.

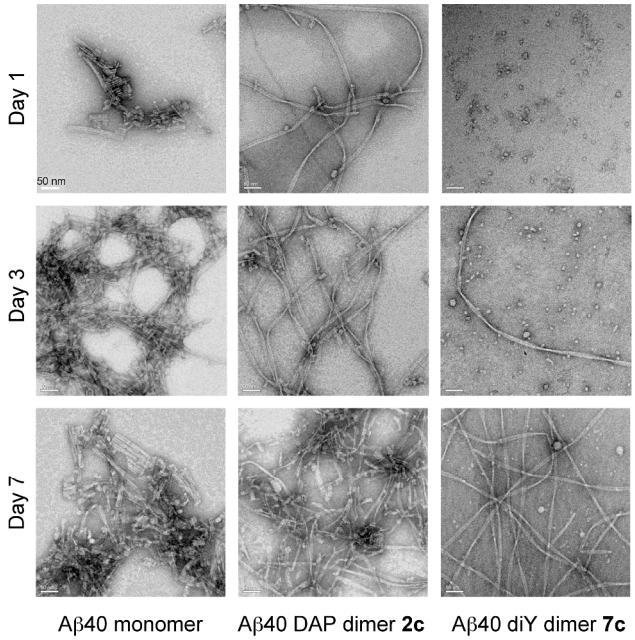

Figure 6. TEM of Aβ40 monomer (left), Aβ40 DAP-linked dimer **2c** (middle) and Aβ40 dityrosine-linked dimer **7c** (right), after aging for 1, 3 and 7 days.

Toxicology

Cell-based assays were undertaken to determine the neurotoxicity of the different Aβ monomer and dimer species. Neuroblastoma SH-SY5Y cells[23] were treated with Aβ monomer species Aβ42, Aβ40 and Aβ40-Met$^{[O]}$35, and the Aβ40 and Aβ40-Met$^{[O]}$35 dityrosine-linked dimers **7c** and **7d**. The peptides were added to the culture medium at 6 μM effective concentration of the Aβ monomer (that is, the monomers were added at 6 μM and the dimers were added at 3 μM). At 6 μM the Aβ40 monomers (containing either Met35 or Met$^{[O]}$35) both have a weak effect on the neuronal cell viability, reducing viability by <10% (Figure 7). In contrast, the Aβ40 dityrosine-linked dimers **7c** and **7d** cause a significant decrease in cell viability (25–30% reduction), comparable to or greater than that of the of the Aβ42 monomer. These results clearly demonstrate that the dityrosine-linked Aβ dimers are significantly more toxic than the corresponding monomers.

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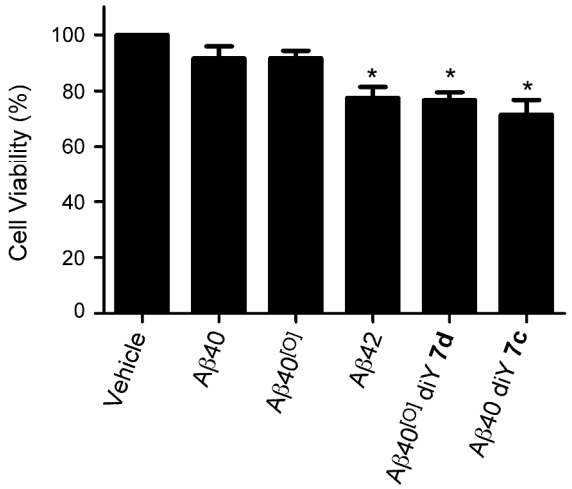

Figure 7. SH-SY5Y neuronal cell viability: cells were treated with 6 μM Aβ peptide for 4 days and cell viability determined as a percentage of the untreated vehicle controls. *, p<0.001 vs vehicle.

Conclusions

In conclusion, we have synthesised for the first time the dityrosine-linked Aβ4 peptide dimers proposed as physiologically-relevant Aβ species present in the brains of AD patients. We have demonstrated that in contrast to other covalently-linked Aβ dimers, the dityrosine-linked dimers display decreased rates of fibrilization and instead form discrete, stable, soluble aggregates. Further, dityrosine-linked Aβ dimers display increased toxicity in a neuronal cell-line assay compared with the corresponding monomers. These biophysical and cell-based assays all provide corroborating evidence that dityrosine-linked Aβ dimers form long-lived, soluble oligomers that are highly neurotoxic – consistent with the hypothesis that dityrosine-linked Aβ dimers are one of the species responsible for the progression of AD. Further studies of the neurotoxicity of Aβ dimers will be reported in due course.

Materials and Methods

Peptide Synthesis and Purification

Dityrosine was prepared according to our reported procedure,[20,21] then converted to Fmoc-protected dityrosine **3** with Fmoc-OSu under standard conditions. Incorporation of Fmoc-dityrosine into the solid phase peptide synthesis (SPPS) strategy for preparation of peptide dimers was performed on Fmoc amino acid loaded PEG-PS resin, using a modified version of our reported method for preparation of DAP-linked Aβ dimers **2**,[9] employing DIC/HOBt in place of HBTU/DIPEA. For the Aβ40 dimer **7c**, cleavage of the peptide was performed in the presence of TMSBr/thioanisole to reduce formation of methionine sulfoxides (*vide infra*).[22] Aβ16, Aβ28 and Aβ40 dimers **7a–d** were purified by the reported methods. Purification of the Aβ42 Met35$^{[O]}$ dimer **7f** was performed by elution through two connecting Superdex^{TM} 75 size exclusion columns (13 μM, 10 mm × 300 mm) with 70% formic acid/30% water with a flow rate of 0.7 mL/min for 35 minutes. Dimer containing fractions were immediately eluted through an HLB Oasis cartridge to remove formic acid with 2% ammonium hydroxide in 90% methanol.

Dynamic Light Scattering

Lyophilised peptides were dissolved on ice under a range of buffer conditions including water alone, 20 mM HEPES pH 7.0, and 1× phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). Samples were subject to centrifugation (15,000 g, 10 min, 4 °C) to remove particulates immediately prior to analysis. DLS measurements were made with a Malvern Instruments Zetasizer Nano ZS instrument. Size distribution profiles were measured repeatedly over the first 30 minutes following dissolution. All samples demonstrated some degree of polydispersity and no discernible difference was observed between buffer conditions. Peptide solutions were prepared at 0.5 mg.mL^{-1}: Aβ16 monomer, 0.26 mM; Aβ16 dimers **2a**, **7a**, 0.13 mM; Aβ28 monomer, 0.15 mM; Aβ28 dimers **2b**, **7b**, 0.08 mM; Aβ40 monomer, 0.11 mM; Aβ40 dimers **2c**, **7c**, 0.06 mM.

ThT assay for fibril formation

Dry peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1.0 mg.mL^{-1}, then aliquotted and dried under vacuum and stored at –80 °C. Peptide concentration was determined using absorbance at 214 nm and extinction coefficients of 91462 M^{-1}cm^{-1} for Aβ40, 239706 M^{-1}cm^{-1} for Aβ40 DAP-dimer **2c** and 197244 M^{-1}cm^{-1} for Aβ40 dityrosine-dimer **7c**, as determined by amino acid analysis. Aliquots of dried peptide were dissolved in 20 mM NaOH then diluted in deionised water and phosphate buffer (100 mM potassium phosphate, pH 7.4) at a v/v/v ratio of 2:7:1. All solutions were sonicated at 0 °C for 10 min and filtered (20 μm) to ensure pre-formed aggregates were removed. Final concentrations: Aβ40 monomer, 14 μM; Aβ40 dimers **2c**, **7c**, 7 μM; Thioflavin-T (ThT), 28 μM; in 1× PBS to a final volume of 600 μL. ThT-induced fluorescence was measured using a fluorescence spectrophotometer fitted with a peltier-driven temperature controller and a multi-cell holder. Each sample was incubated at 37 °C. Excitation was at 444 nm with fluorescence emission measured at 480 nm. Readings were taken every 60 s for the first 15 min, then every 15 min for the next 885 min. Slit widths were 5 nm for both excitation and emission.

Electron Microscopy

A 3.5 μL aliquot of the sample solution used for the ThT assay was adsorbed onto a carbon-coated film mounted on 300-mesh copper grid. Prior to adsorption, the grids were rendered hydrophilic by glow discharge in a reduced atmosphere of air for 10 s. After 30 s adsorption, samples were blotted and negatively stained with 1.5% aqueous uranyl acetate. The transmission electron microscope was operated at 200 kV, with images acquired digitally.

Neuronal cell line toxicity assays

Dry peptide was weighed and dissolved in HFIP to monomerise the peptide, then was then dispensed into small amounts and dried using a speed-vac and stored at –80 °C until use. Aβ peptides were dissolved in 20 mM NaOH, then diluted in water followed by the addition of 1× PBS in a ratio of 2:7:1, as previously described.[1] Peptide concentrations were determined from the absorbance value at 214 nm, using the calculated molar extinction coefficient values of 75887 M^{-1}cm^{-1} for Aβ42, 91462

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$M^{-1}cm^{-1}$ for Aβ40 and Aβ40Met$^{[O]}$, and 197,244 for Aβ40 and Aβ40Met$^{[O]}$ dityrosine dimers **7c** and **7d**.

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 1 $U.mL^{-1}$ of penicillin, 1 $\mu g.mL^{-1}$ of streptomycin, and 2 mM glutamate (Gibco BRL; Invitrogen, Victoria, Australia) and maintained at 37 °C and 5% CO_2 as previously described.[23] To obtain differentiated cells, 20,000 cells were plated per well of a 48-well plate and allowed to adhere for 24 h. Differentiation was started in DMEM supplemented with 1.5% FCS and 10 μM retinoic acid (RA). Fresh medium, containing RA and 1.5% FCS, was applied to the cells every 1–3 days. Experiments were typically performed on cells differentiated for at least 14 days and displaying a differentiated neuronal phenotype, including extensive neurites and branching, as evidenced by light microscopy. To determine the cell toxicity of the Aβ peptides, differentiated cells were treated with 0 and 6 μM effective concentration of Aβ peptides for 4 days in DMEM/1.5% FCS. The cell Counting Kit-8 (Auspep, Australia) was used to determine the cell viability at the end of the treatment. The measured cell viability values were normalised to vehicle-treated control groups (set to 100%) and each treatment was done in at least triplicate (n = 3–7). Results are shown as mean ± S.E.M. The data were analysed by two-tailed Student's t-test. Values of $p<0.05$ were considered significant.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, including the synthesis of **3**, preparation and purification of peptides **7a**–**d**,**f**, and protocols for DLS, ThT, EM and cell viability experiments. See DOI: 10.1039/

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