

Optimization of the Linker Length in the Dimer Model of E22P-A β 40 Tethered at Position 38

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ABSTRACT

Since amyloid β ($A\beta$) oligomers are more cytotoxic than fibrils, various dimer models have been synthesized. We focused on the C-terminal region that could form a hydrophobic core in the aggregation process, and identified a toxic conformer-restricted dimer model (E22P,G38DAP- $A\beta$ 40 dimer) with an L,L-2,6-diaminopimelic acid linker ($n = 3$) at position 38, which exhibited moderate cytotoxicity. We synthesized four additional linkers ($n = 2, 4, 5, 7$) to determine the most appropriate distance between the two $A\beta$ 40 monomers for a toxic dimer model. Each di-Fmoc-protected two-valent amino acid was synthesized from a corresponding dialdehyde or cycloalkene followed by ozonolysis, using a Horner-Wadsworth-Emmons reaction and asymmetric hydrogenation. Then, the corresponding $A\beta$ 40 dimer models with these linkers at position 38 were synthesized using the solid-phase Fmoc strategy. Their cytotoxicity towards SH-SY5Y cells suggested that the shorter the linker length, the stronger the cytotoxicity. Particularly, the E22P,G38DAA- $A\beta$ 40 dimer ($n = 2$) formed protofibrillar aggregates and exhibited the highest cytotoxicity, equivalent to E22P- $A\beta$ 42, the most cytotoxic analog of $A\beta$ 42. Ion mobility mass spectrometry (IM-MS) measurement indicated that all dimer models except the E22P,G38DAA- $A\beta$ 40 dimer existed as stable oligomers (12–24-mer). Native PAGE analysis supported the IM-MS data, but larger oligomers (30–150-mer) were also detected after a 24-h incubation. Moreover, E22P,G38DAA- $A\beta$ 40, E22P,G38DAP- $A\beta$ 40, and E22P,G38DAZ- $A\beta$ 40 ($n = 5$) dimers suppressed long-term potentiation (LTP). Overall, the ability to form fibrils with cross β -sheet structures was key to achieving cytotoxicity, and forming stable oligomers less than 150-mer did not correlate with cytotoxicity and LTP suppression.

KEYWORDS

Alzheimer's disease, amyloid β , dimer, oligomer, SH-SY5Y cells, toxic conformation

INTRODUCTION

Amyloid β proteins ($A\beta$), generated from amyloid precursor protein (APP) by two secretases, are involved in the pathogenesis of Alzheimer's disease (AD).^{1,2} $A\beta$ mainly consists of 40 or 42 amino acid residues ($A\beta_{40}$ and $A\beta_{42}$);^{3,4} the former is 10 times more abundant, but less aggregative and cytotoxic compared to the latter. AD has two pathological characteristics: extracellular amyloid plaques consisting of $A\beta$ fibrils (high molecular weight aggregates) and neurofibrillary tangles of hyperphosphorylated tau proteins in neuronal cells.² In the amyloid cascade hypothesis,⁵ first $A\beta$ aggregates, followed by phosphorylation of tau proteins, ultimately leads to cognitive decline. Aggregation of $A\beta$ begins 20–30 years before the appearance of symptoms, and therefore early diagnosis, followed by lifestyle modifications, is one of the potential preventive responses.

$A\beta$ is prone to self-aggregation because of its highly hydrophobic nature at the C-terminal region,⁶ forming quasi-stable oligomers (e.g., protofibrils),⁷ that eventually become fibrils. At first, $A\beta$ fibrils, a major constituent in senile plaques, were thought to be the cause of cytotoxicity. However, recent studies have shown that soluble $A\beta$ oligomers play a more crucial role than mature $A\beta$ fibrils.^{8–10} Soluble low molecular weight oligomers such as dimers and trimers are the basic units of oligomers. Various $A\beta$ oligomers have been reported, for example, protofibrils,⁷ ADDLs,¹¹ globulomers,¹² and amylospheroids.¹³ These oligomers take different routes to fibrilization; on-pathway and off-pathway.⁹

The cellular membrane is closely involved in the oligomerization of $A\beta$. For example, monosialoganglioside GM1 on the cell membrane surface, plays a crucial role in the aggregation of $A\beta$.¹⁴ $A\beta$ oligomers could bind more strongly to GM1 on neuronal membranes compared with $A\beta$ monomers.¹⁵ Disruption of cell membranes is also linked to cytotoxicity induced by $A\beta$ through membrane fragmentation and pore formation.^{16,17} Recent studies have suggested that intrinsically disordered proteins such as $A\beta$ form complexes with lipids depending on the concentration of free lipids required to make a hole in the membrane.^{18,19} Specifically, for tetramers and octamers of $A\beta_{42}$, the structure of the membrane-associated oligomer with a six-stranded β -sheet core was determined.²⁰ The details of this structure revealed the mechanism of membrane disruption by $A\beta_{42}$.

On the other hand, the chirality of $A\beta$ also affects its oligomerization. For example, D- $A\beta_{42}$ suppresses the oligomerization of L- $A\beta_{42}$ by forming stable fibrils.²¹ In addition, cellular uptake of $A\beta$ depends on its chirality; L- $A\beta_{42}$ was preferably taken up by cells compared to D- $A\beta_{42}$, suggesting that the cellular uptake of $A\beta_{42}$ could be receptor-mediated.²² One of the receptor candidates identified was cellular prion protein (PrP^c).²³

Since $A\beta$ oligomers are in equilibrium with monomers and/or fibrils, isolation of unadulterated oligomeric species is quite difficult. To elucidate the characteristics of $A\beta$

oligomers, various methods have been used including nuclear magnetic resonance (NMR), mass spectrometry, and single particle techniques.^{24–26} Moreover, oligomeric models stabilized by photo-induced cross-linking of unmodified proteins (PICUP) were produced and characterized,²⁷ which contributed to the identification of toxic dimers in the brains of patients with AD.²⁸ On the other hand, many synthetic attempts to obtain stable and chemically pure oligomeric models have also been carried out. For example, disulfide-linked dimer models of A β 40 and A β 42 at position 28 were aggregative, forming fibrillar aggregates with a higher β -sheet content, when compared with their corresponding monomers.²⁹ The disulfide-linked dimer model at position 26 of A β 40 formed protofibrils and decreased long-term potentiation (LTP) in mouse hippocampus.³⁰ A carbon chain-linked dimer model was synthesized by Kok *et al.*³¹ who employed L,L-2,6-diaminopimelic acid (DAP) as a diamino diacid linker (Figure 1). The oligomerization and fibrillization properties of the Y10DAP-A β 40 dimer increased compared to the A β 40 monomer. In contrast, a dityrosine dimer at position 10 of A β 40 formed stable and soluble aggregates with increased cytotoxicity compared to the A β 40 monomer.³² Moreover, disulfide-linked A2C-A β 40 and A β 40-(GGGC) dimer models formed protofibrillar aggregates with high β -sheet content.³³

Our own approach to the toxic dimer models was tethering a DAP linker at position 30 or 38 of E22P-A β 40 as reported previously.³⁴ The E22P mutation made the conformer more toxic by fixing the toxic turn at positions 22/23.^{35–38} The presence of this turn was also observed in the A β 42 aggregates by solid-state NMR^{39–41} and cryo-electron microscopy (Cryo-EM).^{42,43} Another approach using a chiral mutant library of A β 42 also supported the presence of the toxic turn.^{44,45} Among our dimer models, the E22P,G38DAP-A β 40 dimer (**2**) existed as quasi-stable oligomers and exhibited cytotoxicity against SH-SY5Y cells comparable to the E22P-A β 40 monomer.³⁴ It is noteworthy that elongation of the linker at position 30, from DAP ($n = 3$, 3 carbon atoms between each peptide chain) to L,L-2,8-diaminoazelaic acid (DAZ, $n = 5$, 5 carbon atoms), increased the content of the β -sheet structure as exemplified in the E22P,A30DAZ-A β 40 dimer.³⁴

The purpose of this study is to optimize the length of the linker at position 38 of the E22P,G38DAP-A β 40 dimer (**2**). First, we synthesized four additional di-Fmoc-protected two-valent amino acids, L,L-2,5-diaminoadipic acid (DAA; $n = 2$, 2 carbon atoms), L,L-2,7-diaminosuberic acid (DAS; $n = 4$, 4 carbon atoms), DAZ, and L,L-2,10-diaminoundecanedioic acid (DUN; $n = 7$, 7 carbon atoms) (Figure 1). Next, new dimer models, E22P,G38DAA-A β 40 (**1**), E22P,G38DAS-A β 40 (**3**), E22P,G38DAZ-A β 40 (**4**), and E22P,G38DUN-A β 40 (**5**) were synthesized using the solid-phase Fmoc strategy. Here we report their aggregative and oligomer-forming ability, cytotoxicity, and synaptotoxicity, using **2** as a reference.

RESULTS AND DISCUSSION

Synthesis of the Fmoc-linkers and Corresponding Dimer Models of E22P-A β 40 (1, 3–5).

In order to optimize the linker length at position 38 of the E22P,G38DAP-A β 40 dimer (**2**), we synthesized di-Fmoc-protected linkers from dialdehydes or cyclic alkenes by conventional methods (Scheme 1A and Schemes S1–S4 in Supporting Information). Synthesis of di-Fmoc-L,L-DUN is shown in as a typical example (Scheme 1B). Ozonolysis of cycloheptene gave heptanedial (**28**). Horner-Wadsworth-Emmons (HWE) reaction of **28** with (*Z*)- α -phosphonoglycine trimethyl ester yielded the corresponding diolefin (**29**),⁴⁶ whose asymmetric hydrogenation with Rh-QuinoxP* catalyst⁴⁷ gave the chiral amino diacid derivative (**30**) (>99%ee, 96%de, Figure S4). Di-Fmoc-L,L-DUN (**10**) was obtained by hydrolysis, catalytic hydrogenolysis, and Fmoc protection. Di-Fmoc-L,L-DAS (**8**) was synthesized using a similar procedure from 1,5-cyclooctadiene (**16**), while di-Fmoc-L,L-DAA (**6**) and di-Fmoc-L,L-DAZ (**9**) were synthesized from their corresponding dialdehydes, glyoxal (**11**) and pentanedial (**22**), respectively.

Four dimer models with these new linkers at position 38 (**1**, **3–5**) were synthesized with a microwave peptide synthesizer using the Fmoc method.^{35,36} In these syntheses, 0.5 equivalents of each di-Fmoc-linker amino acid were employed to avoid forming a mono-coupled peptide.³⁴ The yields after the HPLC purification were 7.4% (**1**), 5.8% (**3**), 5.3% (**4**), and 4.8% (**5**), respectively. Their purity was determined to be >99% by HPLC, and their molecular weights and formulae were verified by HR-ESI-qTOF-MS (Figure S5).

Cytotoxicity of Dimer Models 1–5 against SH-SY5Y Neuroblastoma Cell Lines. The cytotoxicity of dimer models **1–5** against SH-SY5Y cells was assessed by the MTT assay. Although the MTT assay has various limitations that affect the results, such as the number of seeding cells, incubation time in the presence of A β , and culture media composition, it is commonly used to estimate cytotoxicity related to cellular metabolic activity.⁴⁸ After incubation for 24 h in the presence of each dimer model or E22P-A β 40, cell viability was evaluated by the ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as reported previously.³⁸ As shown in Figure 2A, E22P-A β 40 as a positive control significantly decreased cell viability at 3.3 μ M. Consistent with the previous report,³⁴ dimer model **2** with a DAP linker ($n = 3$) was more cytotoxic than E22P-A β 40. Although the intermolecular β -sheet is most specifically simulated by a DAZ linker ($n = 5$),³⁴ cytotoxicity of **4** with a DAZ linker was unexpectedly weak. Dimer model **5** with a DUN linker ($n = 7$) was inactive even at 10 μ M, and cytotoxicity of **3** with a DAS linker ($n = 4$) was weaker than **2**. These results indicate that an increase of the linker length at position 38 proportionally decreased the cytotoxicity.

Consistent with this trend, the cytotoxicity of **1** with a DAA linker ($n = 2$) was very strong; it reached maximum neurotoxicity at 1 μ M. As shown in Figure 2B, the cytotoxicity of **1** was almost equal to that of E22P-A β 42, the most cytotoxic analog of A β 42 except for those with an intramolecular disulfide bond at positions 17/28,^{36,38} and was *ca.* 30 times more potent compared with the E22P-A β 40 monomer. These results indicate that tethering at the C-terminal region of A β 40 could drastically increase the cytotoxicity to become comparable to that of A β 42 derivatives. This is the first dimer model of A β 40 that exhibited potent cytotoxicity comparable to A β 42.

Ability to Restrain Long Term Potentiation (LTP). In response to the MTT assay, we assessed LTP alteration using the dimer models and E22P-A β 40 as a positive control in mouse hippocampal slices. LTP is widely used as a neural model of the cellular mechanism involved in learning and memory. Recent investigations^{8,11,49} reported that soluble oligomers including dimers inhibited LTP, while trimers could be more potent inhibitors compared with dimers and tetramers.⁵⁰

LTP was elicited in the hippocampal CA1 region by high-frequency stimulation (HFS) after recording baseline activity for 40 min (Figure 3A). Hippocampal slices were exposed to vehicle (artificial cerebrospinal fluid; ACSF), each dimer model, or E22P-A β 40, 20 min after the start of recording. Slices exposed to the vehicle exhibited strong LTP induction after HFS. Dimer models **1**, **2**, and **4** along with E22P-A β 40 significantly inhibited LTP to a similar extent, while inhibition by **5** was very weak and not significant (Figure 3B). These results basically correlated with those of cytotoxicity against SH-SY5Y cells (Figure 2). However, the area under the curve (AUC) was almost the same among **1**, **2**, **4**, and E22P-A β 40 (Figure 3B). It might be necessary to decrease the concentration of each compound below 10 nM to distinguish the potency of LTP inhibition more accurately. It is also noteworthy that the effective concentration for LTP inhibition (less than 10 nM) was significantly lower than causing cytotoxicity (0.1–10 μ M), suggesting that a different mechanism exists.

Aggregative Ability by Th-T and TEM Analyses of Dimer Models 1–5. The aggregative ability of dimer models **1–5** along with wild-type (WT)-A β 42, E22P-A β 42, and E22P-A β 40 as references was assessed using thioflavin-T (Th-T),⁵¹ which was fluorescent when bound to A β aggregates with a β -sheet structure (Figure 4). Each dimer model (10 μ M) was incubated with Th-T (20 μ M), and Th-T fluorescence was automatically monitored every 10 min at room temperature (23 °C) as reported previously.^{38,52} WT-A β 42 and E22P-A β 42 aggregated rapidly to reach maximum fluorescence after 4 h. The difference in the maximum fluorescence intensity might reflect the fibril structure to which Th-T is bound. E22P-A β 40 started to aggregate after 8

h and reached almost maximum fluorescence after 16 h, followed by an incremental increase. Dimer model **1** with the shortest linker length ($n = 2$) started to aggregate after 10 h and exhibited the highest fluorescence among dimer models **1–5**. Dimer models **2** and **3** showed weak fluorescence, and **4** and **5** did not display any. The aggregation velocity of each dimer model was slow compared with the monomers, and none of the dimer models (10 μ M) aggregated to reach the half-maximum fluorescence of E22P-A β 40 (20 μ M).

In order to investigate the morphology of each aggregate, transmission electron microscopy (TEM) analysis was carried out (Figure 5). Each sample was analyzed after a 48-h incubation at 37 °C as reported previously.³⁸ WT-A β 42 and E22P-A β 40 were used as references and formed typical amyloid fibrils. Among dimer models **1–5**, only **1** formed typical amyloid fibrils similar to E22P-A β 40 and WT-A β 42. Some fibrillar aggregates (protofibrils) were observed for **2** with a DAP linker ($n = 3$). Our previous data also reported that **2** formed protofibrillar aggregates.³⁴ Dimer models **3–5** did not form a clear structure; instead, they formed irregularly shaped aggregates. These data suggest that only dimer models **1** and **2** with short linkers ($n = 2$ and 3) formed fibrils or protofibrillar aggregates. These results suggest that the ability to form fibrils or protofibrillar aggregates could be important for potent cytotoxicity.

Secondary Structure of Dimer Models 1–5 Based on CD Spectra. Since dimer models **3–5** did not form fibrillar aggregates, circular dichroism (CD) measurements were conducted to obtain their secondary structural information (Figure 6). As a positive control, E22P-A β 40 exhibited a negative peak at ~ 200 nm after 0 h, and a positive peak at ~ 200 nm and a negative peak at ~ 220 nm appeared after a 6-h incubation at 37 °C. This suggests that the initial random structure changed to the β -sheet-rich structure during incubation. Dimer model **1** exhibited a similar spectrum, especially a positive peak at ~ 200 nm and a negative peak at ~ 220 nm even after a 2-h incubation. This suggests that **1** formed a β -sheet-rich structure more rapidly compared with E22P-A β 40. Since the increase of cross β -sheet structures detected by Th-T fluorescence (Figure 4) correlated with cytotoxicity (Figure 2), this structural change of **1** could be related to its potent cytotoxicity against SH-SY5Y cells.

From their CD spectra, the structure of dimer models **2–5** was expected to contain fewer β -sheets than E22P-A β 40 and **1**. Intensity of the negative peaks at ~ 220 nm was smaller than that of **1**, and the peak top shifted to a lower wavelength. It seems that these dimer models adopt multiple conformations with an α -helix or β -sheet-rich structure.

Estimation of Oligomerization of Dimer Models 1–5 by IM-MS and Nondenaturing PAGE. In order to determine the size of oligomers of dimer models **1–5**, ion mobility-mass spectrometry (IM-MS) measurement was carried out. IM-MS combined with native ionization

techniques and ion mobility spectrometry could separate each multivalent ion on two-dimensional spectra. Applications of IM-MS to the analyses of A β oligomers have been reported.^{53–55} The two-dimensional heat maps with m/z and drift time after a 4-h incubation are shown in Figure 7. Enlarged views are also shown in the Supporting Information (Figures S6–S10). There is a linearity in the two-dimensional heat map among similar oligomeric structures with a different charge.⁵⁶ The linearity of each oligomer suggests that these oligomers have similar conformations with different charges. However, there were several peaks not easily assigned, suggesting the existence of oligomers with multiple conformations and/or tertiary structures.

A 4-h incubation was necessary for sufficient oligomer formation with maximum molecular size as reported previously.³⁴ After extensive analyses based on the observed masses, peaks corresponding to oligomeric orders were assigned to the series of multiply charged ions depending on their drift time (Figures S11–S15). Dimer models **2–5** showed quasi-stable oligomers of 12–24-mer ($n = 6–12$). A spectrum similar to that reported previously³⁴ was obtained for **2** with a DAP linker, suggesting that this measurement has a high reproducibility. The reason why these dimer models existed mainly as 12–24-mer is difficult to explain; however, the process of the formation of a 12-mer A β 17-36 fragment has been reported.⁵⁷ According to the report, three monomers aggregate to form a triangular structure, which subsequently assembles into a tetrahedron to form a dodecamer. Finally, five dodecamers pack together to form an annular pore, which induces cytotoxicity. In contrast, only low molecular weight oligomers ($n = 1–6$) were observed for **1** with a DAA linker. These results were unexpected since the formation of quasi-stable oligomers of 12–24-mer oligomers was thought to be responsible for the potent cytotoxicity of A β .³⁴

According to our previous data,^{34,38,58} identification of the peaks greater than 30-mer with IM-MS was quite difficult because of their weak intensity and peak broadening due to the number of isotopes. It is likely that **1** formed oligomers greater than 30-mer since quasi-stable oligomers of E22P-A β 42, the most aggregative and cytotoxic analog of A β 42,^{59,60} could not be identified by IM-MS.⁶¹ In order to assess the oligomerization of the dimer models, polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions was carried out (Figure 8).

Each dimer model (1.0 μ M) or each A β monomer (2.0 μ M) was incubated in phosphate-buffered saline for 24 h at 37 °C. The molecular size was assessed under nondenaturing conditions by blue-native gel electrophoresis immediately after dissolution and after a 24-h incubation as reported previously.^{37,38} As shown in Figure 8, WT-A β 42 and E22P-A β 40 did not show any significant signals ascribable to quasi-stable oligomers less than 1,000 kDa; only the signal of high molecular weight oligomers including protofibrils was observed. This indicates that these monomers aggregated rapidly without staying as quasi-stable oligomers. Dimer model **1** with potent cytotoxicity (Figure 2) behaved like WT-A β 42 and E22P-A β 40 with significant

cytotoxicity; few quasi-stable oligomers were detected after 0-h and 24-h incubations (Figure S16 in Supporting Information). In contrast, dimer models **3–5** existed as quasi-stable oligomers (66–140 kDa) immediately after dissolution, and these oligomers shifted to larger molecular weight oligomers (>140 kDa) after a 24-h incubation. However, these dimer models exhibited faint Th-T fluorescence (Figure 4), suggesting that Th-T detects preferably highly aggregated fibrils with a cross β -sheet structure such as those of WT-A β 42 and E22P-A β 40 (Figure 5). The relatively low content of a β -sheet structure of the dimer models **3–5** suggested by CD spectra (Figure 6) could also support the Th-T data. Dimer model **2** gave both high molecular weight aggregates and quasi-stable oligomers after a 24-h incubation. IM-MS data of **2** and **3** after 0-h and 24-h incubations (Figures S17 and S18) correlated with these results; 12–24-mer oligomers were detected in both compounds. Similar IM-MS data were observed for **4** and **5** after a 0-hr incubation (Figures S19 and S20).

Overall, the present results using various E22P-A β 40 dimer models tethered at position 38 suggested that the formation of quasi-stable oligomers less than 700 kDa (~150-mer) is not a requisite condition for cytotoxicity. Similar conclusions have been reported by Raskatov and colleagues using the chiral mutant library of A β 42,^{44,45} and by our team using conformation-restricted analogues of A β 42 with an intramolecular disulfide bond.³⁸ Formation of larger oligomers like protofibrils and/or the aggregation process itself, and not the specific oligomers, could be critical to inducing potent cytotoxicity as proposed previously.^{62,63}

CONCLUSIONS

In summary, we synthesized four additional dimer models (**1**, **3–5**) based on the previous model with a DAP linker (**2**, $n = 3$),³⁴ to optimize the linker length of dimer models cross-linked at position 38 (Figure 1). Among the dimer models, **1** with a DAA linker ($n = 2$) was most cytotoxic against SH-SY5Y cells, and the cytotoxicity was comparable to that of E22P-A β 42, the most cytotoxic analog among the proline mutants of A β 42.^{59,60} The structure–activity relationship was that the shorter the linker length, the more cytotoxic the dimer. This suggests that the tethering at position 38 of A β 40 could substitute for the two C-terminal hydrophobic residues of A β 42 and induce cytotoxicity, but that the length of the linker is important for inducing cytotoxicity. A similar tendency has quite recently been observed in the parallel-type trimer model of E22P-A β 40 at position 38.⁶⁴ In this case, DAP-type trimer model was cytotoxic against SH-SY5Y cells, but the DAZ-type trimer model was not.

The present study also suggested that the formation of large oligomers like protofibrils is indispensable to the mechanism of cytotoxicity. In other words, cytotoxicity of oligomers less than 700 kDa (150-mer) is weak. However, this conclusion may not apply to the inhibition of

LTP since **4** with a DAZ linker ($n = 5$) significantly inhibited LTP at 10 nM (Figure 3). Further studies are necessary to determine the effective concentration of the dimer models to inhibit LTP and identify the molecular size of toxic oligomers for LTP inhibition. It is possible that the A β oligomers on the pathway toward stable fibrils could also induce cytotoxicity. It is also noteworthy that the increase in cross β -sheet structures correlated with cytotoxicity rather than the ability to form stable oligomers less than 150-mer.

Since **1** was extremely cytotoxic among the dimer models synthesized thus far, it could be a basic lead compound in the design of ideal dimer models, and useful to study the mechanism of toxicity by A β dimers. However, caution is warranted because **1** is an E22P mutant of A β 40, which is not present in the brain. This mutation could also affect its interaction with the cellular membrane, GM1, metal ions, and other amyloid proteins (cross-seeding).⁶⁵

MATERIALS AND METHODS

General Remarks. HR-ESI-qTOF-MS was carried out with Waters Xevo G2-S qTOF (Waters, Milford, MA, USA). Peptide synthesis was conducted by Initiator+ Alstra (Biotage, Uppsala, Sweden). The transmission electron microscope used was JEM-1400 (JEOL, Tokyo, Japan). CD spectra were recorded on J-805 spectropolarimeter (Jasco, Tokyo, Japan). Preparative HPLC was carried out using a Model 600E pump with a Model 2487 UV detector (Waters, Milford, MA, USA). Analytical HPLC was performed with a Model 1525 binary HPLC pump with a Model 2489 UV/Visible detector and EmpowerTM3 software (Waters, Milford, MA, USA).

Materials for the synthesis of di-Fmoc-DAA, DAS, DAZ, and DUN (Supporting Information) were obtained from Tokyo Chemical Industry (Tokyo, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and other commercial suppliers, and used without further purification.

HATU⁶⁶ was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). *N,N*-Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Foster City, CA, USA). Fmoc amino acids were purchased from Watanabe Chemical Industries (Hiroshima, Japan), Applied Biosystems (Foster City, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and other commercial suppliers. *N,N*-Dimethylformamide (DMF), trifluoroacetic acid (TFA), ethane-1,2-dithiol, thioanisole, *m*-cresol, and diethyl ether (peroxide free) were purchased from Nacalai Tesque (Kyoto, Japan). Piperidine was obtained from Sigma-Aldrich (St. Louis, MO, USA) and Watanabe Chemical Industries (Hiroshima, Japan).

Synthesis of di-Fmoc-DAA, DAS, DAZ, and DUN (Figure 1). The synthetic procedures are described in Supporting Information.

Synthesis of Dimer Models. The E22P-A β 40 dimer models (**1**, **3–5**) were synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc L-Val TentaGelTM PHB resin (loading: 0.20 mmol/g) supplied by Rapp Polymere (Tübingen, Germany) using the Fmoc method. One-half of a molar equivalent of each dimer

linker was used instead of Fmoc-L-Gly at position 38 in order to avoid the formation of the mono-coupled peptides. Each coupling reaction was carried out using each Fmoc amino acid (0.4 mmol, 0.5 M in DMF), HATU (0.4 mmol, 0.5 M in DMF), and DIPEA (0.8 mmol, 1.0 M in DMF) for 5 min at 75 °C with microwave irradiation. The coupling reaction of histidine was carried out for 10 min at 50 °C to avoid racemization. The coupling reaction of each dimer linker (di-Fmoc-DAA, DAS, DAZ, or DUN) was conducted for 10 min at 75 °C. After each coupling reaction, the N-terminal Fmoc group was deblocked with 20% piperidine in DMF.

After the completion of chain elongation followed by Fmoc deblocking, the resultant peptide resin was washed with DMF and CH₂Cl₂, and shaken for 2 h at room temperature in a mixture containing TFA (16 mL), *m*-cresol (0.4 mL), 1,2-ethanedithiol (1.2 mL), and thioanisole (2.4 mL) for final deprotection and cleavage from the resin. Each crude peptide was precipitated by diethyl ether at 4 °C. A portion of the crude peptide was purified using HPLC on a YMC Pack PROTEIN-RP column (20 mm I.D. × 150 mm, 5 μm, YMC CO., LTD, Kyoto, Japan) with elution at 8.0 mL/min by an 80-min linear gradient (curve 6) of 20–60% CH₃CN containing 0.1% TFA. Subsequent purification was carried out using an XBridge Peptide BEH C18 OBDTM Prep Column (10 mm I.D. × 250 mm, 300 Å, 5 μm, Waters, Milford, MA, USA) with elution at 3.0 mL/min by a 60-min exponential gradient (curve 7) of 20–50% CH₃CN containing 0.1% TFA. E22P,G38DAP-Aβ40 dimer (**2**) which was synthesized previously,³⁴ was only purified using the HPLC conditions above. Lyophilization produced each peptide, the purity of which was checked by HPLC (>99%, Figure S5). Molecular weights and formulae were confirmed by HR-ESI-qTOF-MS measurements (Figure S5).

MTT Assay Using SH-SY5Y Cells. MTT assay was carried out using the method reported previously.^{34,38} The human neuroblastoma SH-SY5Y cell line (ATCC, Manassas, VA, USA), which is used as a neuronal cell model to estimate the cytotoxicity of Aβ peptides, was maintained in a 1:1 mixture of Eagle's minimum essential medium and Ham's F12 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) and 1% MEM non-essential amino acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). As reported previously, each Aβ was dissolved in 0.15% NH₄OH to give a 110 μM stock solution. The stock solution was diluted with 0.15% NH₄OH to the appropriate concentrations (0.36, 1.1, 3.6, 11, 36 μM) and each 10 μL aliquot was subsequently added to 100 μL of the culture medium containing near-confluent cells (2 × 10⁴ cells/well) to give final concentrations of 0.033, 0.1, 0.33, 1, 3.3, and 10 μM. After being treated at 37 °C for 24 h, 15 μL/well of Dye solution in CellTiter 96[®] Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was added to the cells, followed by a 4-h incubation at 37 °C. The solubilization/stop solution (100 μL/well) was subsequently added to the cells. Next, the resultant cell lysate was incubated overnight in the dark at room temperature before performing measurements at 570 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). Absorbance obtained by the addition of vehicle (0.15% NH₄OH) was taken as 100%.

Hippocampal Slice Electrophysiology Recording (Inhibition of LTP). Male C57BI/6Ncr mice (Japan SLC, Shizuoka, Japan) 6–7 weeks old were used to prepare acute hippocampal slices. All experimental procedures involving the use of animals were approved by the University of Toyama Animal Experimentation Committee (approval number: A2018PHA-23, A2021PHA-14), and were in accordance with the Guidelines of the Japanese Pharmacological Society. The inhibition of LTP was measured by the same method reported previously.³⁸

Thioflavin-T (Th-T) Assay. Th-T fluorescence was measured automatically based on the previously described procedure with slight modifications.³⁸ For instance, 390 μ L of 50 mM sodium phosphate and 100 mM NaCl (PBS, pH 7.4) were aliquoted into a 1.5 mL tube and 10 μ L of 1 mM Th-T solution was added. Each A β was dissolved in 0.15% NH₄OH at 100 μ M (E22P-A β 40) or 50 μ M (dimer models). The 100 μ L of each A β solution was added to the tube of PBS/Th-T solution to become a final concentration of 20 μ M (E22P-A β 40) or 10 μ M (dimer models). The samples were aliquoted into a 96-well black plate (100 μ L/well) and incubated at room temperature (23 °C). Fluorescence intensity was measured automatically every 10 min at 430 nm excitation and 485 nm emission using a microplate reader (Fluoroskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA). For each assay, a vehicle control without A β solution was subtracted as blank to calculate substantial fluorescence.

Transmission Electron Microscopy (TEM). The aggregates of each A β solution (WT-A β 42, E22P-A β 40, 25 μ M; dimer models 1–5, 12.5 μ M) in 50 mM sodium phosphate and 100 mM NaCl (pH 7.4) after a 48-h incubation were examined under a transmission electron microscope as reported previously³⁸ (JEM-1400, JEOL, Tokyo, Japan). After each A β aggregate was centrifuged (4 °C, 15,000 rpm, 10 min), the supernatant was removed from the pellet. The resultant pellet was gently resuspended in water (WT-A β 42, E22P-A β 40: 100 μ L, dimer models 1–5: 50 μ L) using a vortex for 1 min. The suspension (15 μ L) was applied to a 400-mesh carbon-coated copper grid (thickness: 15–20 nm; Veco, Eerbeek, Netherlands) and allowed to incubate for 5 min before being negatively stained twice with 2% uranyl acetate. Stained samples were subsequently subjected to TEM.

Circular Dichroism (CD) Spectrometry. CD spectra were measured using a 0.1 mm quartz cell. Each A β was dissolved in 0.15% NH₄OH at 250 μ M (E22P-A β 40) or 125 μ M (dimer models). They were diluted 10 times with 50 mM sodium phosphate and 100 mM NaCl (pH 7.4) to a final concentration of 25 μ M (E22P-A β 40) or 12.5 μ M (dimer models) and incubated at 37 °C. After 0, 2, 4, 6, 24, and 48 h, an aliquot (200 μ L) was loaded into a cell, and the CD spectrum was recorded at 190–260 nm after subtracting the spectrum of the vehicle.

Ion Mobility-Mass Spectrometry (IM-MS). IM-MS was measured by the method reported previously.^{34,58} Each dimer model was dissolved in 0.2% NH₄OH at 125 μ M, followed by a 10-fold dilution with 25 mM ammonium acetate before incubation for 4 h at 37 °C. The incubated sample (12.5 μ M) was centrifuged for 5 min at 18,000 g before infusion into the MS apparatus using a glass capillary (Nanoflow Probe Tip, Waters, Milford, MA, USA). Mass spectra and ion mobility experiments were conducted on

SYNAPT XS HDMS (Waters, Milford, MA, USA) using a nano electrospray as an ionization source. The instrument was operated in negative ion mode with a capillary voltage of 0.7–1.0 kV, a sample cone voltage of 150 V, and a source temperature of 50 °C. For the ion mobility measurement, nitrogen gas was used in an ion mobility cell, and the cell pressure was maintained at approximately 2.83 mbar with a wave velocity of 650 m/s and a wave height of 40 V. Data acquisition and processing were performed with the MassLynx (V4.2) and DriftScope (V3.0) software supplied with the instrument. The CsI cluster ions were used for m/z scale as a calibrator.

Nondenaturing PAGE. In order to observe the aggregates of the dimer models under nondenaturing conditions (without detergents or reducing agents), we used a method based on the Blue-native PAGE (BN-PAGE) technique.^{37,38} A β peptides prepared at 400 μ M in dimethylsulfoxide were diluted to make final concentrations (WT-A β 42, E22P-A β 40: 2 μ M, dimer models **1–5**: 1 μ M) in phosphate-buffered saline (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4). The samples were incubated at 37 °C for 24 h. Then, 7.5 μ L of each sample was mixed with 2.5 μ L of NativePAGE 4 $\times$ sample buffer (Thermo Fisher, Waltham, MA, USA), and 3.75 μ L of NativeMark unstained protein standard (Thermo Fisher, Waltham, MA, USA) mixed with 1.25 μ L of NativePAGE 4 $\times$ sample buffer was loaded onto a NativePAGE 4–16%, Bis-Tris gel (Thermo Fisher, Waltham, MA, USA) and electrophoresed for 30 min at 150 V using the dark-blue running buffer provided by the manufacturer. At that time, proteins had migrated through one-third of the gel length. Buffer was then exchanged for a light-blue running buffer. The gel was further electrophoresed at 250 V for 60 min. After electrophoresis, proteins were transferred to the PVDF membrane (Immobilon-P, Merck Millipore, Burlington, MA, USA). Membranes were blocked in 5% skim milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were incubated with 82E1 antibody⁶⁷ (IBL, Gunma, Japan) at 0.3 μ g/mL in blocking buffer for 16 h at 4 °C. On the following day, membranes were washed for 10 min $\times$ 3 with TBST and incubated 1 h in HRP-labeled anti-mouse immunoglobulin G (IgG) (Thermo Fisher, Waltham, MA, USA) at 1:5000 in TBST. Membranes were washed for 10 min $\times$ 3 with TBST and exposed to Chemi-Lumi One chemiluminescent substrate (Nacalai Tesque, Kyoto, Japan). The chemiluminescent signal was recorded with ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxxxxxxx.

The Supporting Information contains the synthetic schemes and procedures of dimer linkers **6** and **8–10**; ^1H and ^{13}C NMR spectra of new compounds; HPLC profiles for ee and de determination of intermediates of the dimer linkers; HPLC profiles and HR-ESI-qTOF-MS data of dimer models **1** and **3–5**; detailed IM-MS data of dimer models **1–5**; and projections of 2D spectra and the calculated and observed masses of dimer models **1–5** (PDF).

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Author Contributions

K.I. designed the project. A.C. and K.I. analyzed and interpreted the experiments. A.C. and A.U. synthesized the dimer linkers under the guidance of C.T. A.C. synthesized the dimer models under the guidance of K.I. and Y.I. A.C. performed the Th-T and TEM analysis. Y.I. performed the MTT assay and non-denaturing PAGE analysis. T.K. and K.H. performed the IM-MS measurements. Y.K. and I.T. supervised the non-denaturing PAGE. A.C., Y.I. and K.I. drafted the manuscript, and K.I., C.T. and I.T. reviewed the manuscript.

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Notes

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ABBREVIATIONS

AD, Alzheimer's disease; A β , amyloid β ; CD, circular dichroism; Cryo-EM, cryo-electron microscopy; DAA, L,L-2,5-diaminoadipic acid; DAZ, L,L-2,8-diaminoazelaic acid; DAP, L,L-2,6-diaminopimelic acid; DAS, L,L-2,7-diaminosuberic acid; DUN, L,L-2,10-diaminoundecanedioic acid; IM-MS, ion mobility-mass spectrometry; NMR, nuclear magnetic resonance; Th-T, thioflavin-T; TEM, transmission electron microscopy.

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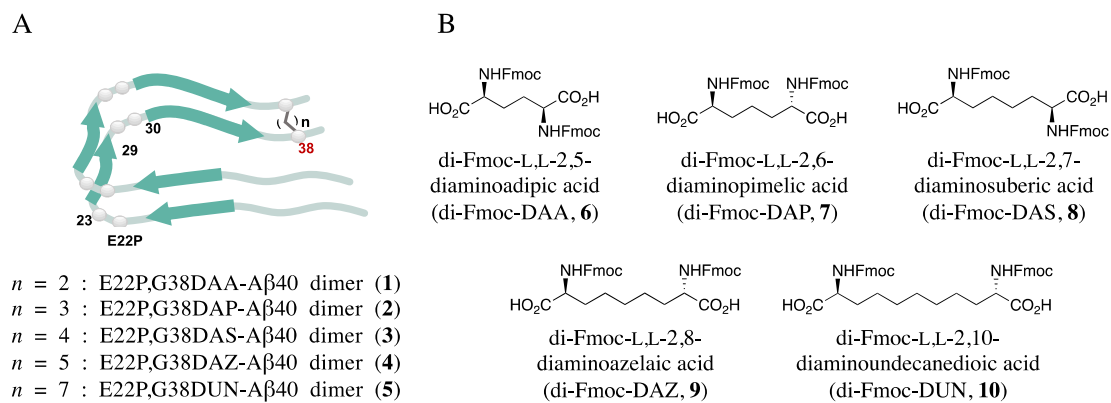


Figure 1. (A) Structures of A β 40 dimer models (**1–5**). (B) Structures of linkers (**6–10**).

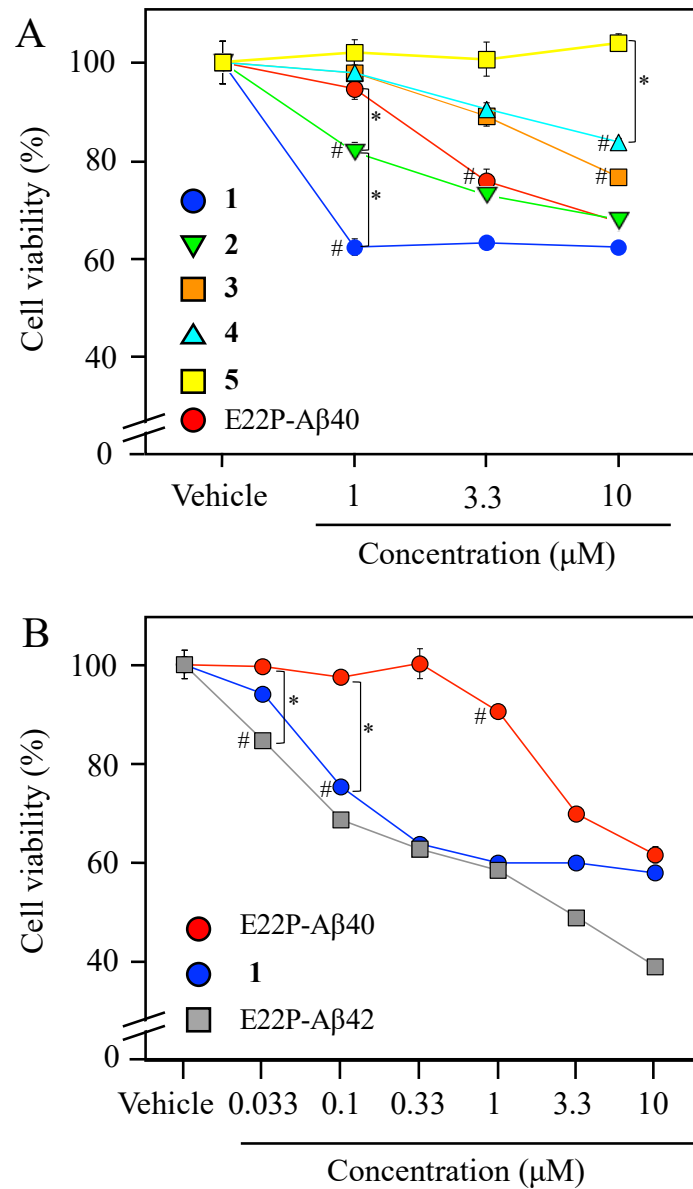


Figure 2. (A) Cytotoxicity of dimer models 1–5 and E22P-Aβ40 towards SH-SY5Y cells after a 24-h incubation at 37 °C. (B) Cytotoxicity of the dimer model 1, E22P-Aβ40, and E22P-Aβ42 towards SH-SY5Y cells after a 24-h incubation at 37 °C. Absorbance obtained after adding vehicle (0.15% NH₄OH) was taken as 100%. **p* < 0.001, #*p* < 0.001 versus vehicle. Data are expressed as the mean ± SD (*n* = 3).

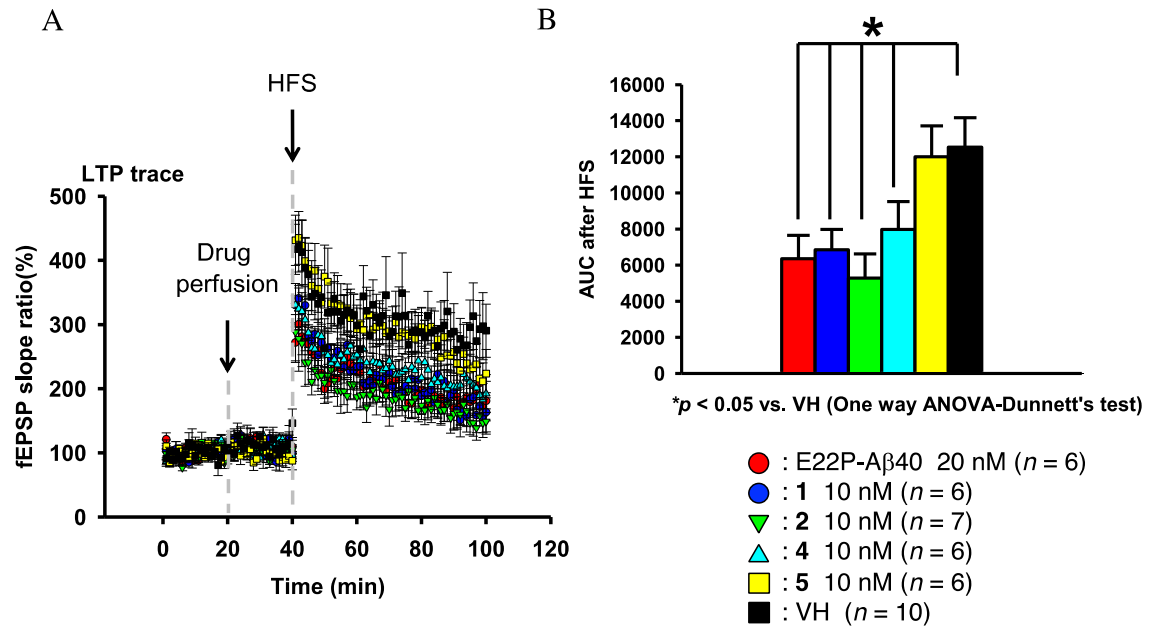


Figure 3. (A) LTP induction in mouse hippocampal slices treated with **1** (DAA), **2** (DAP), **4** (DAZ), and **5** (DUN) at 10 nM and E22P-A β 40 at 20 nM. The concentration of E22P-A β 40 was doubled because it is a monomer. The mean standard field excitatory postsynaptic potentials (fEPSP) slope of the first 20 min was taken as 100%. Data are presented as mean $\pm$ SEM. (B) The area under the curve (AUC) versus baseline after induction of LTP by HFS for the vehicle (ACSF), **1**, **2**, **4**, **5** and E22P-A β 40. The values of fEPSP slope after inducing LTP were integrated after substitution of baseline fEPSP slope for each subject. Data are presented as the mean $\pm$ SEM. * $p < 0.05$ versus vehicle.

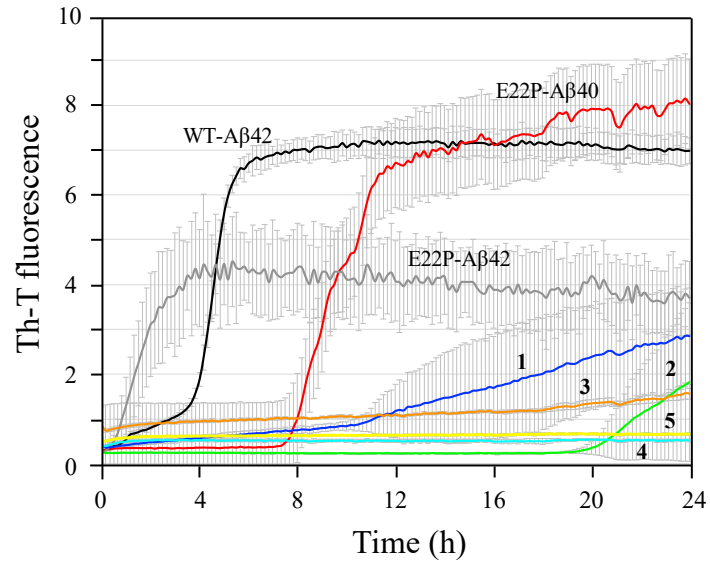


Figure 4. Th-T fluorescence of dimer models **1-5** (10 μ M) and WT-A β 42 (10 μ M), E22P-A β 40 (20 μ M), and E22P-A β 42 (10 μ M). The concentration of E22P-A β 40 was doubled because it is a monomer. Th-T fluorescence of 42-mer A β was significantly higher than that of 40-mer A β under these conditions. The concentration of the A β 42 peptides was thus set at half that of E22P-A β 40. Data are expressed as the mean $\pm$ SD ($n = 4$).

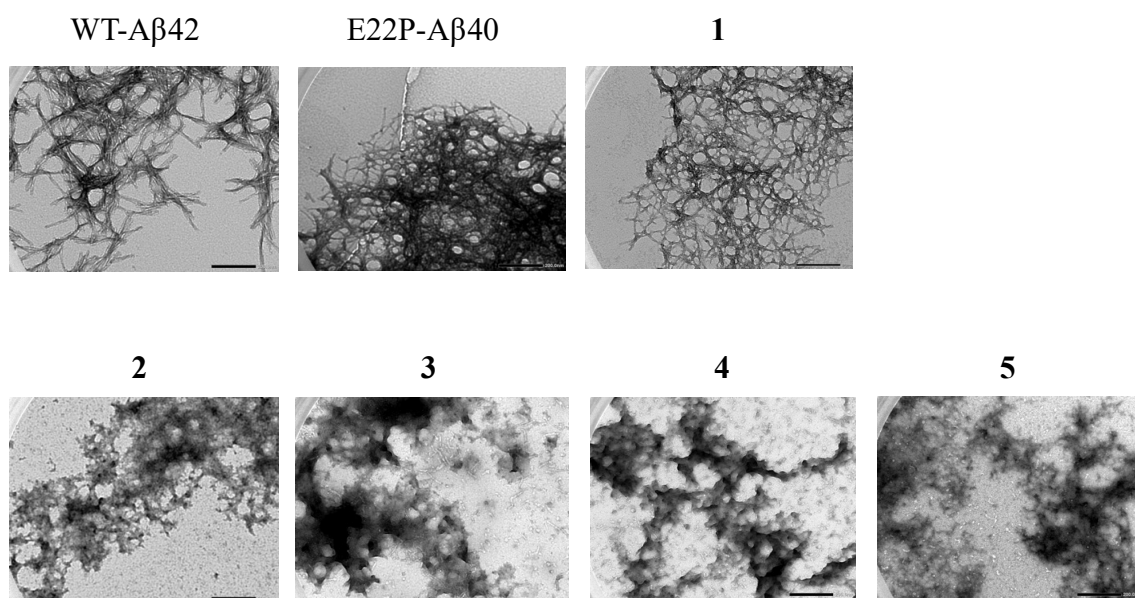


Figure 5. TEM analyses of the aggregates of dimer models **1–5** (12.5 μ M), WT-A β 42 and E22P-A β 40 (25 μ M) after a 48-h incubation at 37 °C. The concentration of E22P-A β 40 was doubled because it is a monomer. Scale bar = 200 nm (magnification: 30 k).

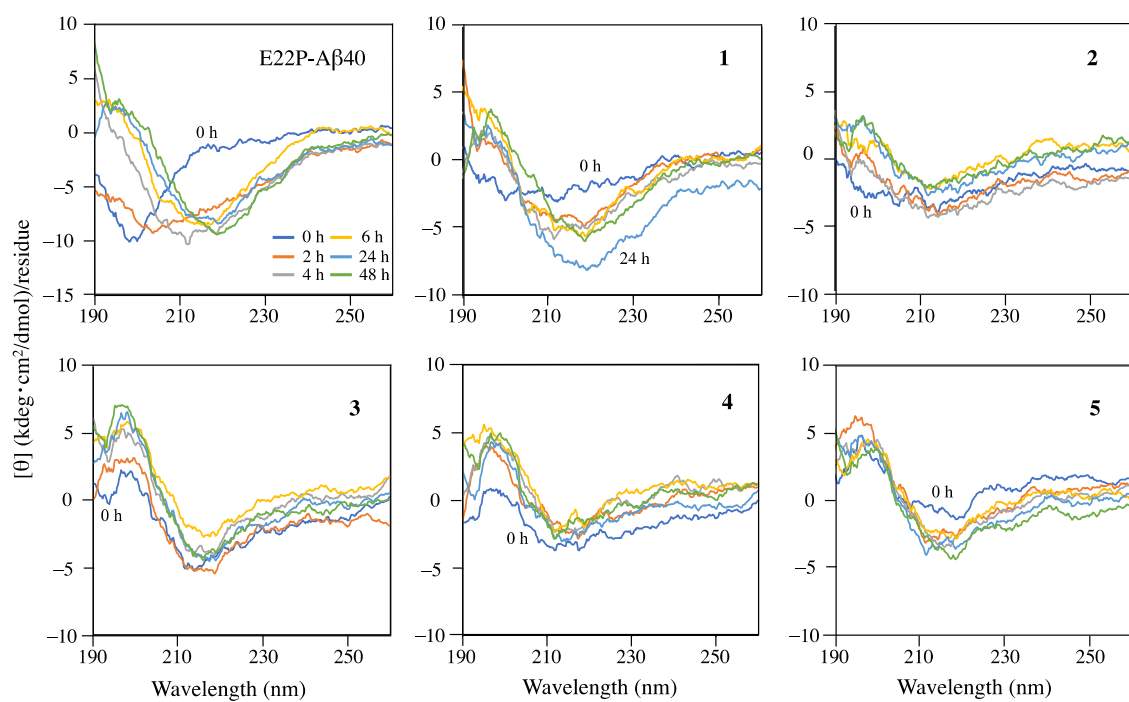


Figure 6. CD spectra of dimer models 1–5 (12.5 μM) and E22P-A β 40 (25 μM) after incubation for the indicated duration at 37 $^{\circ}\text{C}$. The concentration of E22P-A β 40 was doubled because it is a monomer.

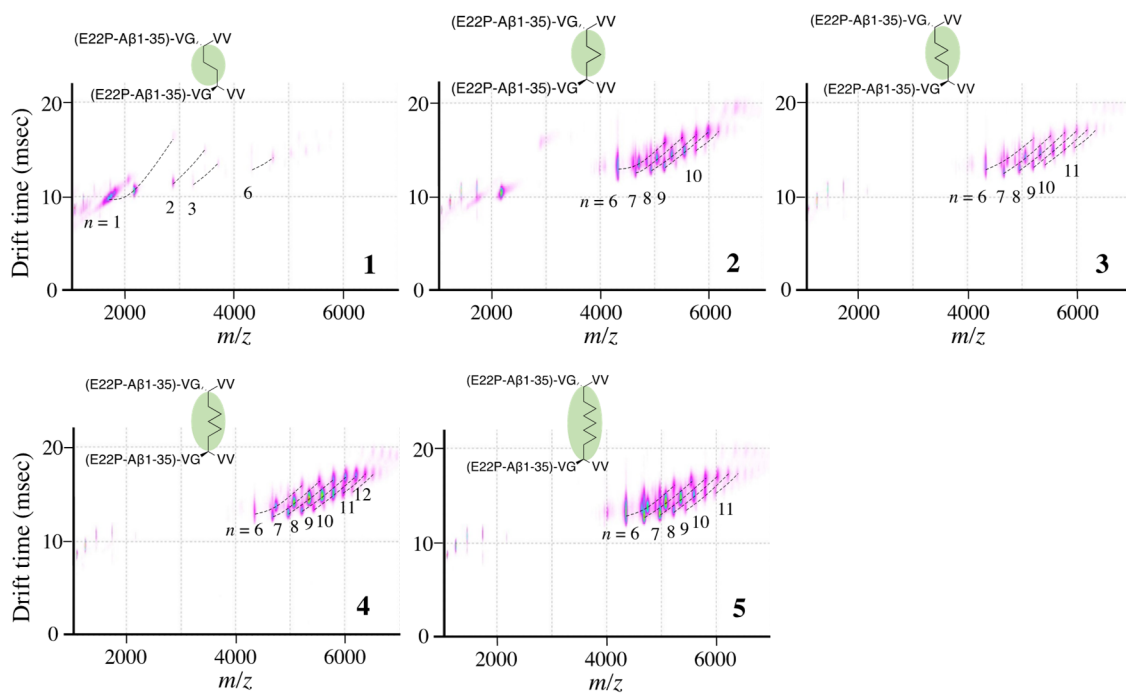


Figure 7. IM-MS of dimer models 1–5 (12.5 μ M) after a 4-h incubation at 37 °C. “ n ” denotes an integer corresponding to the number of units coexisting in the solution [$n = 1, 2, 3, \dots$ of a dimer model denotes dimer, tetramer, hexamer, ..., respectively] depending on their drift time. The signal amplitude with linear scale is color-coded, increasing from purple (low intensity) to blue (high intensity).

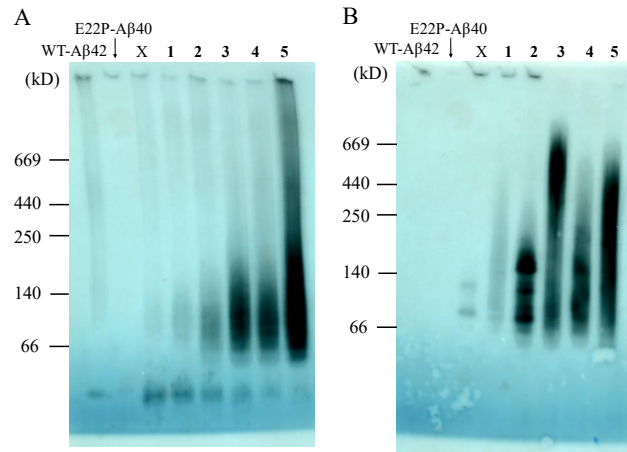
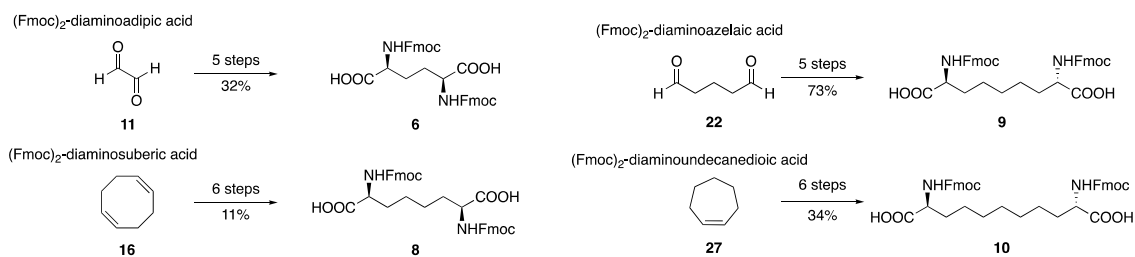
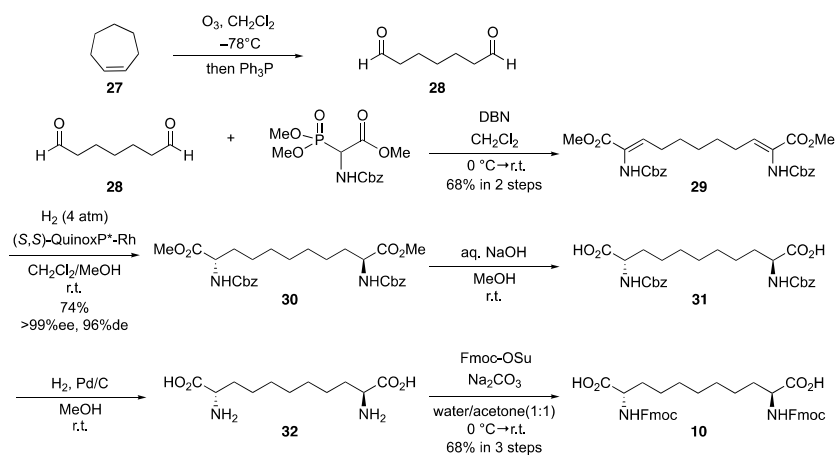


Figure 8. Non-denaturing PAGE of dimer models 1–5 (1 μ M), WT-A β 42, and E22P-A β 40 (2 μ M). The concentration of E22P-A β 40 was doubled because it is a monomer. The concentration of each sample was reduced to 1/10 of the Th-T, CD, and IM-MS analyses to mimic the concentration of the MTT assay (Figure 2) because the sensitivity of antibody was high. (A) A 0-h, (B) a 24-h incubation at 37 °C. X: Void.

A



B



Scheme 1. Synthesis of di-Fmoc-protected diamino acids. (A) Summary. (B) Synthesis of di-Fmoc-L,L-2,10-diaminoundecanedioic acid (DUN). Detailed synthetic procedures are described in the Supporting Information (Schemes S1–S4).

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