Synthesis and characterization of the amyloid β40 dimer model with a linker at position 30 adjacent to the intermolecular β-sheet region

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Abstract

Amyloid fibrils in senile plaque mainly consist of the 40-mer and 42-mer amyloid β-proteins (Aβ40 and Aβ42). Although Aβ42 plays more important role in the pathogenesis of Alzheimer's disease (AD), Aβ40 could be involved in the progression of AD pathology because of its large amount. Recent studies revealed that variable sizes of Aβ oligomers contributed to the neuronal death and cognitive dysfunction. However, how large oligomeric species are responsible for AD pathogenesis remains unclear. We previously proposed a toxic dimer model of Aβ with turn structure at positions 22 and 23 using solid-state NMR and systematic proline replacement. Based on this model, we herein show the synthesis and biological activities of an E22P-Aβ40 dimer at position 30, which was connected to L,L-2,6-diaminopimeric acid. The E22P-Aβ40 dimer formed stable 6~8-mer oligomers without amyloid fibrils, but was not neurotoxic on human neuroblastoma cells. On the other hand, E22P-Aβ40 generated high molecular-weight oligomers into fibrils, and showed the neurotoxicity. These results suggest that such kind of Aβ40 dimer with a parallel β-sheet might not be pathological.

Highlights

- E22P-Aβ40 dimer with turn at positions 22 and 23 was synthesized.
- The cross-linkage at position 30 by optically active L,L-DAP was adopted in the dimer.
- E22P-Aβ40 dimer formed 6~8-mer oligomers without fibrils, but was not neurotoxic.
- Aβ40 dimer with a parallel β-sheet might not be related to Alzheimer's pathology.

Keywords

Alzheimer's disease, amyloid β-protein, dimer, oligomer, neurotoxicity.

Abbreviations

Aβ, amyloid β; AD, Alzheimer's disease; L,L-DAP, L,L-2,6-diaminopimeric acid; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; TEM, transmission electron microscopy; Th-T, thioflavin-T.

1. Introduction

In Alzheimer's disease (AD), amyloid fibrils form senile plaques that mainly consist of 40- and 42-residue amyloid β-proteins (Aβ40 and Aβ42) [1]. Aβ42 is regarded as the leading cause of AD because its aggregative ability and neurotoxicity are more potent than those of Aβ40 [2]. In contrast, Aβ40 could be involved in the progression of AD pathology because of its large amount. Accumulating evidence has shown that the soluble oligomeric assembly of Aβ is more exclusively implicated in neuronal death and cognitive dysfunction than insoluble fibrils and protofibrils [3,4]. "Aggregation" in this context is defined as the change from Aβ monomers into amyloid fibrils *via* oligomers and protofibrils. Since Aβ42 only weakly associates with Aβ40 [5,6], homogeneous oligomers of Aβ42 or Aβ40 form more easily than the corresponding heterogeneous oligomers. The minimum unit of these oligomers, which have been divided into low molecular-weight oligomers (2~12-mer) and high molecular-weight oligomers (24~100-mer), is considered to be a dimer or trimer (2 or 3 x *n*-mer) [7]. However, how large oligomeric species of Aβ are responsible for the pathogenesis of AD remains unknown.

Our previous study using solid-state NMR [8] and systematic proline replacement [9] identified a toxic conformer of Aβ42 with a turn structure at Glu22 and Asp23, in which the residues at positions Gln15~Ala21 and Val24~Ile32 took part in the intermolecular parallel β-sheet of Aβ42 aggregates. Moreover, the C-terminal hydrophobic core formed by another turn at Gly38 and Val39 together with the intramolecular β-sheet (Met35~Gly37 and Val40~Ala42) accelerated the aggregation (oligomerization) of Aβ42 [10]. Based on these findings, we proposed a structural model for a toxic dimer of Aβ42 with a turn at Glu22 and Asp23 as shown in Figure 1A. Regarding the aggregates of Aβ40, the most abundant species among Aβ [2], both Aβ40 and Aβ42 would have the turn structure at positions 22 and 23 together with the intermolecular parallel β-sheet, whereas the C-terminal hydrophobic core is absent in the Aβ40 aggregates because of its lower hydrophobicity based on Wetzel's model [11].

Practical synthesis of Aβ dimers have thus far been limited to Aβ40 dimers due to the

intrinsic potent aggregative ability of Aβ42 dimers during the synthesis and preparation. For example, Kok *et al*. [12] reported the synthesis of a dityrosine cross-linked Aβ42 dimer at position 10; however, its biological activity was not tested due to the insufficiently low amounts obtained. Based on the elevated levels of dityrosine-linked Aβ dimers in the brains and blood of AD patients, Kok *et al*. synthesized two dimer models of Aβ40, in which dityrosine [12] or bifunctional 2,6-diaminopimeric acid (DAP) as "a diastereomeric mixture" [13] was incorporated at position 10 instead of Tyr10. Both models facilitated the production of fibrils in spite of the formation of globular aggregates [12,13] associated with neurotoxicity of the dityrosine-linked Aβ40 dimer [12]. The dityrosine-linked Aβ40 dimer synthesized by Walsh and co-workers also formed typical amyloid fibrils, even though it impaired long-term potentiation in rats *in vivo* [14]. The disulfide-linked Aβ40 dimer at Ser26 inhibited long-term potentiation [15,16], but did not appear to be physiologically relevant to the pathology of AD.

Since Aβ40 dimers connected adjacent to the intermolecular β-sheet region have not yet been examined, we synthesized a new dimer model of E22P-Aβ40 (Fig. 1B) with the toxic turn, in which position 30 was linked to L,L-2,6-diaminopimeric acid (L,L-DAP: Fig. 1C), not to the diastereomeric mixture [13], in order to investigate the effects of dimerization on its biological activity. Wild-type Aβ40 was negligibly neurotoxic, whereas E22P-Aβ40 was neurotoxic, albeit 10-fold less than wild-type Aβ42 [17]. Ala30 was selected as the linking position because our previous solid-state NMR studies using rotational resonance (R2) revealed that this residue in E22K-Aβ42 aggregates (Italian mutant) was proximal (< 6 Å) [18].

2. Material and methods

2.1. Synthesis of the E22P-Aβ40 dimer.

L,L-DAP (Fig. 1C) was synthesized according to the protocol established by Paradisi *et al*. [19] with slight modifications [20]. L,L-DAP was obtained from (*S*)-α-phenylethylamine in 6 steps (2.5 % yield). The structure of L,L-DAP was confirmed

by EI-MS, HR-FABMS (JMS-600H; JEOL, Tokyo, Japan), and ^{1}H NMR (AVANCE III 400 and 500; Bruker, Rheinstetten, Germany, ref. DHO = 4.79 ppm, D_2O) : δ 1.46 (2H, m), 1.91 (4H, m), 3.77 (2H, t, *J* = 5.9 Hz) ppm. The specific rotation (P-2200; Jasco, Tokyo, Japan) of L,L-DAP was similar to that reported previously; $[\alpha]_D$: + 37.6°(*c* 0.96, 1N HCl, 28 ºC), ref. $[\alpha]_D$: + 42.1°(*c* 0.96, 1N HCl) [19]. The amino groups of L,L-DAP were protected with Fmoc for the solid-phase synthesis of the E22P-Aβ40 dimer; ^{1}H NMR (ref. CD_2HOD = 3.31 ppm, CD_3OD): δ 1.54 (2H, m), 1.74 (2H, m), 1.88 (2H, m), 4.16 (4H, m), 4.30 (4H, m), 7.28 (4H, m), 7.34 (4H, t, *J* = 7.0 Hz), 7.63 (4H, m), 7.75 (4H, d, *J* = 7.4 Hz) ppm.

The E22P-Aβ40 dimer (Fig. 1B) was synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-L-Val-PEG-PS resin by Pioneer™ (Applied Biosystems; Foster City, CA, USA) using the Fmoc method, as described elsewhere [21]. Briefly, after the completion of chain elongation and cleavage from the resin, the crude peptide was precipitated by diethylether, followed by purification using HPLC on a YMC-Pack PROTEIN-RP column (20 mm i.d. x 150 mm; YMC, Kyoto, Japan) with elution at 8.0 mL/min by a 70-min linear gradient of 20-60% CH_3CN containing 0.1% trifluoroacetic acid. Subsequent purification was carried out using YMC-Pack ODS-A (20 mm i.d. x 150 mm; YMC) with elution at 8.0 mL/min by an 80-min linear gradient of 20-60% CH_3CN containing 0.1% trifluoroacetic acid. Lyophilization gave a pure peptide, the purity of which was confirmed by HPLC (>98%, 2.8% yield). The molecular weight of the E22P-Aβ40 dimer was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker Ultraflex); *m/z*, calculated: 8608.80; found: 8609.55 $[MH]^+$.

2.2 Thioflavin-T (Th-T) assay.

The aggregative ability of each Aβ was evaluated by thioflavin-T (Th-T; Sigma, St. Louis, MO, USA) fluorescence assay developed by Naiki and Gejyo [22]. The procedure was described elsewhere [21]. Each Aβ was dissolved in 0.1% NH_4OH at 250 μM, followed by 10-fold dilution with phosphate buffered saline (PBS: 50 mM sodium

phosphate and 100 mM NaCl, pH 7.4) at the final concentration of 25 μM. After incubation at 37 ºC for the desired period, 2.5 μL of the reaction solution was added to 250 μL of 5.0 μM Th-T in 5.0 mM Gly-NaOH (pH 8.5), followed by the measurement of fluorescence at 430 nm excitation and 485 nm emission using a microplate reader (Fluoroskan Ascent; Thermo Scientific, Rockford, IL, USA).

2.3. Transmission electron microscopy (TEM).

The aggregates of each Aβ were examined under a H-7650 electron microscope (Hitachi, Ibaraki, Japan). The experimental procedure was described elsewhere [21]. After the supernatant was removed from pellets obtained in Th-T assay, resultant aggregates were then suspended in water (20 μL) by gentle vortex mixing, and centrifuged 13,000 rpm for 3 min. These suspensions were applied to a 200 mesh formvar-coated copper grid (Nissin EM, Tokyo, Japan), and allowed to dry in air for 5 min before being negatively stained for 2 min with 2% uranyl acetate, and subsequently subjected to microscope.

2.4. Size exclusion chromatography.

Each Aβ solution, as prepared in Th-T assay, was incubated at 37ºC. After the solution was collected temporally and centrifuged at 15,000 rpm at 4ºC for 10 min, 60 μL of the supernatant was analyzed by size exclusion chromatography on the Superdex75 10/300GL column (10 mm i.d. x 300 mm; GE Healthcare, Fairfield, CT, USA) with elution at 0.6 mL/min by filtered- and degassed-PBS, attached to a Waters LC system with a 2489 UV/Visible detector and 1525 binary HPLC pump controlled by Empower™3 software (Waters, Taunton, MA, USA). The peptide was detected by absorbance at 220 nm. Calibration curves of size exclusion columns were constructed by dextran standards (Mp: mean peak molecular weight, 43,500; 21,400; 9,890; 4,440 Da) (Sigma) together with Blue dextran 2000 (GE Healthcare) as an indicator of the void volume (V_o).

2.5. MTT assay.

SH-SY5Y cells, maintained in Dulbecco's modified eagle medium (DMEM; Wako, Osaka, Japan) containing 10% fetal bovine serum, were used as one of the typical neuronal cell models to estimate the neurotoxicity of each Aβ with slight modifications to the described method [21]. In brief, each Aβ was dissolved in 0.1% NH_4OH to 220 μM. The resultant peptide solution (10 μL) diluted with 0.1% NH_4OH to appropriate final concentrations (10^{-7}, 10^{-6}, $10^{-5.5}$, 10^{-5} M) in the medium was added to 100 μL of the culture medium of near-confluent cells (10^4 cells/well). After being treated at 37°C for 48 hr, 10 μL of 5 mg/mL MTT (Sigma) was added to cells, followed by an incubation for 4 h at 37°C. After the removal of 70 μL of the culture medium, the cell lysis buffer (100 μL/well; 10% SDS, 0.01 M NH_4Cl) was subsequently added to the cells. The cell lysate was then incubated overnight in the dark at room temperature before measurements at 595 nm with a microplate reader (MultiScan JX; Thermo Scientific, Waltham, MA, USA). Absorbance obtained by the addition of vehicle (0.1% NH_4OH) was taken as 100%.

2.6. Statistical analysis.

All data are presented as the mean ± SEM. The differences were analysed with one-way analysis of variance (ANOVA), followed by Bonferroni's test or unpaired Student's *t*-test. These tests were implemented within GraphPad Prism software (version 5.0d). p values <0.05 were considered significant.

3. Results

3.1. Synthesis of the E22P-Aβ40 dimer

After several trials and errors, we adopted L,L-DAP (Fig. 1C) as a covalent cross-linker of E22P-Aβ40 with significant neurotoxicity, albeit 10-fold less than wild-type Aβ42 [17]. Solid-phase synthesis using Fmoc-L,L-DAP at position 30, adjacent to the intermolecular β-sheet region of Aβ40 aggregates, provided a pure and sufficient amount of the E22P-Aβ40 dimer as shown in Fig. 1B. (purity: >98%, 2.8% yield). Given E22P mutant as a toxic conformer surrogate, a monoclonal antibody (11A1) [23], previously developed

against the toxic conformer of Aβ with turn at positions 22 and 23, reacted with the E22P-Aβ40 dimer (data not shown). We further attempted to synthesize a E22P-Aβ40 dimer doubly cross-linked at positions 17 and 30 using L,L-DAP in order to enhance the stability of the toxic conformation of E22P-Aβ40 dimer, but such an attempt was disappointing (data not shown).

3.2. The ability of the E22P-Aβ40 dimer to form fibrils

The aggregative ability of the E22P-Aβ40 dimer with a linker at position 30 was estimated using Th-T, a reagent that fluoresces when bound to Aβ aggregates, and transmission electron microscopy (TEM). As shown in Figure 2A, E22P-Aβ40 aggregated with a lag time of ~8 h and a maximum fluorescence value after being incubated for 24 h. The wild-type Aβ40 did not aggregate after a 24-h incubation under the same conditions, as reported previously [21]. In contrast, the fluorescence of the E22P-Aβ40 dimer remained almost unchanged even after a 336-h incubation (14 days) (Fig. 2A). These results were consistent with TEM results showing that globular aggregates, not fibrils, were predominantly detected in the E22P-Aβ40 dimer (Fig. 2B). On the other hand, the typical amyloid fibrils together with high molecular-weight oligomeric aggregates were formed in E22P-Aβ40 even after 2 days (Fig. 2B). These results indicated that the E22P-Aβ40 dimer had the ability to form a soluble assembly and globular oligomers, but not fibrils.

3.3. The ability of the E22P-Aβ40 dimer to form oligomers

Oligomerization of the E22P-Aβ40 dimer was analysed further using size exclusion chromatography. As a control reference, Figure 3A showed that the soluble peak corresponding to the monomer of E22P-Aβ40 time-dependently decreased, and almost disappeared after an incubation for 16 h; this result implied that E22P-Aβ40 directly formed insoluble fibrils, as observed in Figure 2B. On the other hand, the E22P-Aβ40 dimer formed stable oligomers of 6~8-mer during incubation for 4~48 hr (Fig. 3B).

Although the assembly size of the predominant oligomers after 168-h and 336-h incubations exceeded 10-mer, it was difficult to calculate this correctly because of the limitation of the size exclusion column (Superdex75 10/300GL) used in this experiment.

3.4. The neurotoxicity of the E22P-Aβ40 dimer on SH-SY5Y cells

The neurotoxicity of the E22P-Aβ40 dimer in human neuroblastoma SH-SY5Y cells, one of the typical models of neuronal cell cultures, was measured using the MTT assay. After being incubated for 48 h, the viability of cells treated with E22P-Aβ40 (3.2 μM = $10^{-5.5}$ M) significantly decreased, which indicated E22P-Aβ40 to be neurotoxic as reported previously in PC12 cells [21] (Fig. 4A). In contrast, the neurotoxicity of the E22P-Aβ40 dimer was not observed, even at 10 μM ($10^{-5.5}$ M), as shown in Fig. 4B. Ono *et al.* prepared a mixture of dimers, trimers, and tetramers of wild-type Aβ40, using photo-induced cross-linking of unmodified protein (PICUP) technology that mainly bound covalently at Tyr10 [24], and demonstrated that each oligomer of Aβ40 exhibited the neurotoxicity in a size-dependent manner [25]. However, the marginal toxicity of the PICUP-induced Aβ40 dimer did not seem to basically contradict our results in Figure 4B.

4. Discussion

We demonstrated that the dimer model of E22P-Aβ40 (Fig. 1B) linked at position 30 adjacent to the intermolecular β-sheet region in the aggregates formed stable low molecular-weight oligomers, mainly consisting of 6~8-mers, that did not extend to fibrils. However, this dimer model was less neurotoxic than the corresponding E22P-Aβ40 monomer with the toxic turn at positions 22 and 23, suggesting that such kind of the dimer model in Figure 1B, which is the minimum structure of non-toxic Aβ40 fibrils [26], could not be crucially involved in the pathogenesis of AD. Given the significant toxicity of E22P-Aβ40, the formation of high molecular-weight oligomers in the case of Aβ40 may be required for AD pathology. Although the insignificant toxicity of the E22P-Aβ40 dimer does not necessarily deter further investigations on the position of cross-linking, the

synthesis and characterization of dimer models of "more toxic Aβ42" appear to be more important. Whether high molecular-weight oligomers or low molecular-weight oligomers derived from Aβ42 can contribute to the neurotoxicity will be an attractive issue in the future. Since this study validated optically active L,L-DAP as a useful linker near the intermolecular β-sheet region of Aβ40, this strategy may also be applicable to the synthesis of various Aβ42 dimer models.

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Figure legends

Fig. 1. Toxic dimer models of Aβ42 and Aβ40 with turns at positions 22 and 23. (A) A toxic dimer model of Aβ42 based on our previous structural studies using solid-state NMR and systematic proline replacement. (B) The E22P-Aβ40 dimer covalently cross-linked at Ala30 by L,L-DAP, whose structure was based on Wetzel's model [11]. (C) L,L-DAP as a molecular linker

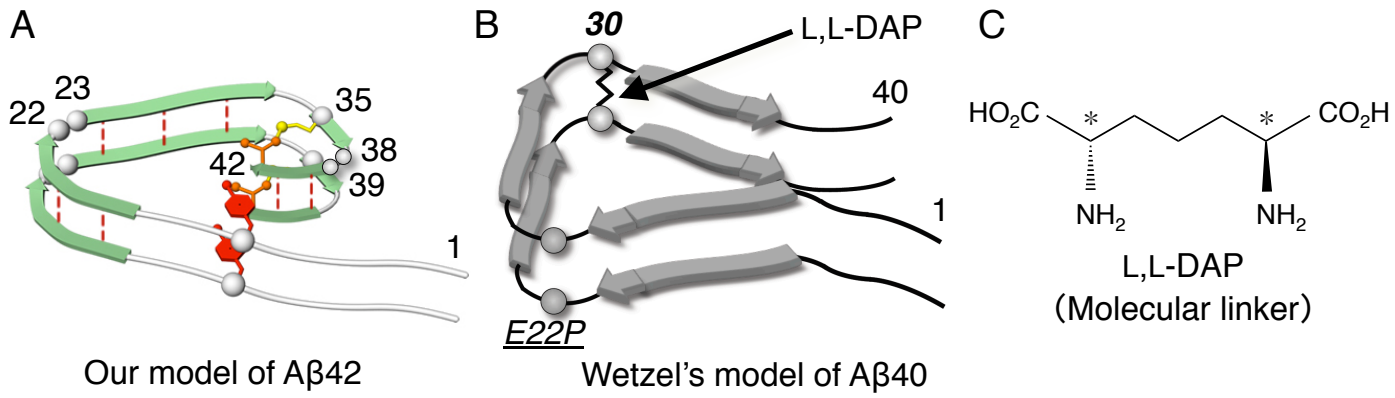
Fig. 2. Analyses of the aggregative abilities of the E22P-Aβ40 monomer and dimer to form fibrils. (A) The Th-T assay of each Aβ derivative after incubation of the indicated period. ●, E22P-Aβ40; ○, the E22P-Aβ40 dimer. Data were presented as the mean ± SEM (n = 8). (B) A TEM analysis of Aβ aggregates formed from E22P-Aβ40 and the E22P-Aβ40 dimer after incubation of the indicated period. Scale bar = 100 nm. *Left*, E22P-Aβ40; *Right*, the E22P-Aβ40 dimer.

Fig. 3. Analyses of the abilities of the E22P-Aβ40 monomer and dimer to form soluble oligomers. The ability of each Aβ derivative to form soluble oligomers was evaluated by size exclusion chromatography after incubation of the indicated period. (A) E22P-Aβ40; (B) the E22P-Aβ40 dimer. The peptide was detected by absorbance at 220 nm. The size of molecular marker (kDa) is shown. V_o: void volume. *: artifact.

Fig. 4. Neurotoxicity of the E22P-Aβ40 monomer and dimer in SH-SY5Y cells. The near-confluent SH-SY5Y cells (10^4 cells/well) were treated with the E22P-Aβ40 monomer or dimer (10^{-7}, 10^{-6}, $10^{-5.5}$, 10^{-5} M) in DMEM medium containing 10% fetal bovine serum before incubation at 37°C for 48 hr. Data were presented as the mean ± SEM (n = 3). Absorbance obtained by the addition of vehicle (0.1% NH_4OH) was taken as 100%. *p< 0.05 *versus* vehicle (Veh).

Figure

Fig. 1. Murakami, K. *et al*.



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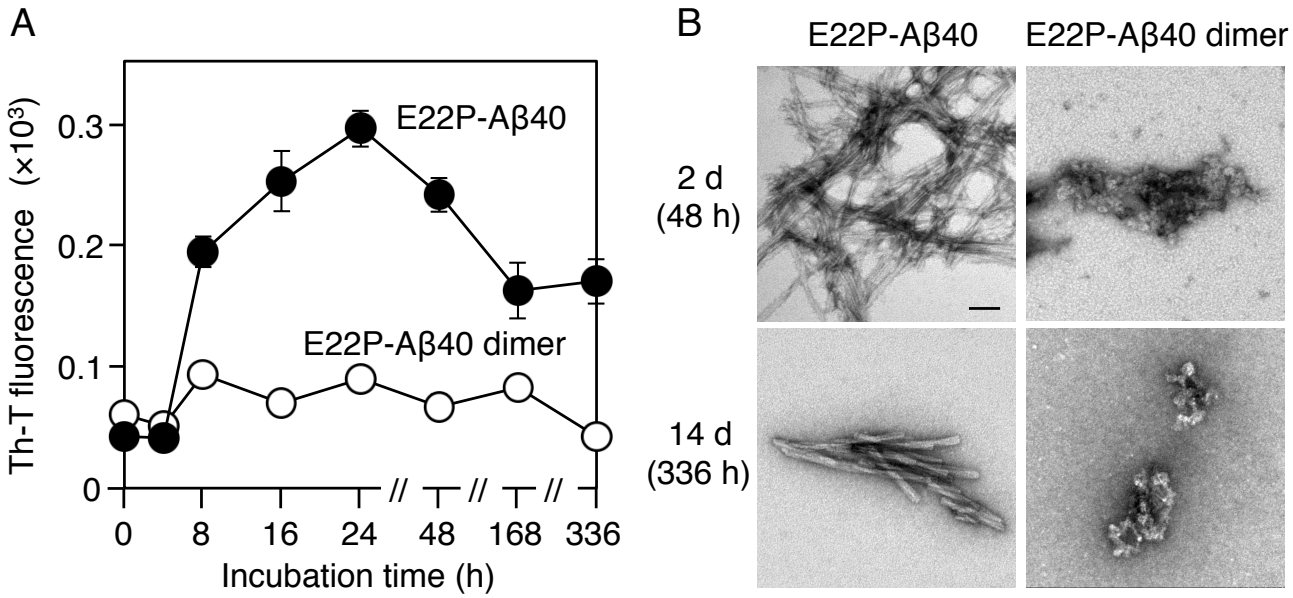

Fig. 3. Murakami, K. *et al*.

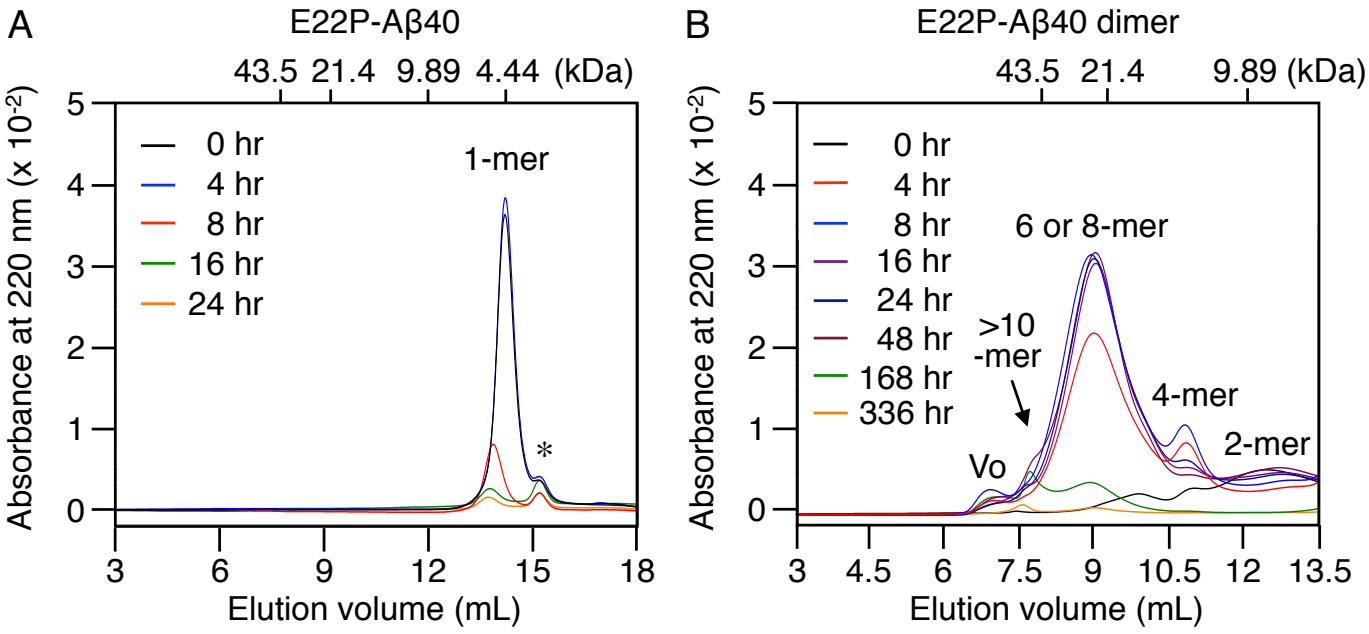
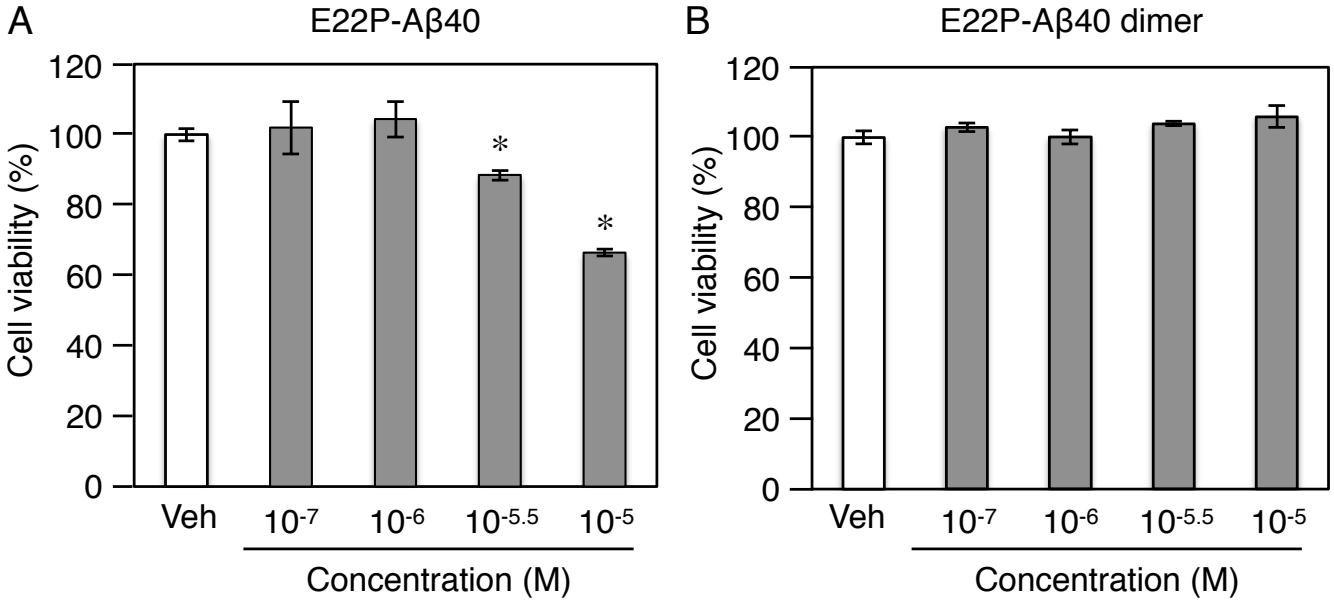
Fig. 4. Murakami, K. *et al*.