Chemically-engineered, stable oligomer mimic of amyloid β42 containing an oxime switch for fibril formation

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Chemically-engineered, stable oligomer mimic of amyloid β42 containing an oxime switch for fibril formation

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Toxic aggregation of monomeric amyloid β (Aβ) into oligomers followed by the formation of fibrils is a causative process in the pathogenesis of Alzheimer's disease. The mechanism for furnishing the toxicity of Aβ aggregates is elusive, however, mainly due to the transient, unstable properties of the oligomer states. Oligomer mimics stabilized by chemical protein engineering are potentially useful tools for elucidating the pathogenicity of Aβ aggregates. Here we report a stable Aβ oligomer mimic that is transformed into fibrils by a chemical stimulus, i.e., an oxime exchange reaction. A derivative of Aβ42[Met^{35}(O)], compound **2** containing an oxime tether between residues 23 and 28 (a salt-bridge surrogate between Asp^{23} and Lys^{28} of Aβ42 oligomer), rapidly and homogeneously formed stable, relatively large oligomers with preserved amyloid-like properties, such as the propensity to form β-sheets and toxicity. Chemical cleavage of the tether via an oxime exchange reaction induced transformation of the oligomers into the fibril state. These results demonstrate that the oxime bond formation/cleavage can switch the aggregation state of the mimic by functionally surrogating the salt-bridge of Aβ42. This novel system temporally dissects the dynamic process of Aβ aggregation, and thus might offer a unique molecular tool for exploring the properties of Aβ oligomers and fibrils.

Introduction

Amyloid fibrils with an extensive β-sheet structure (a so-called cross-β-sheet), generated through protein aggregation, cause a number of diseases.[1] Amyloid β (Aβ), mainly comprising 40- or 42-residue amino acids, is a representative amyloid protein (peptide). Aβ fibrils accumulate in the brains of patients with Alzheimer's disease, and constitute senile plaques.

Aβ fibrils are the end-products in the aggregation process, and are thus stable and isolable. In contrast, Aβ oligomers are intermediates of the aggregation process, and are therefore transient and unstable. Accordingly, investigating the properties of the oligomers through this dynamic process is difficult. Utilization of oligomer mimics stabilized by chemical protein engineering is a valuable approach to investigate the properties of transiently existing oligomers. Indeed, several stabilized Aβ oligomer mimics have been reported, including; 1) Aβ derivatives comprising double-cysteine mutants that form an intramolecular disulfide bond,[2a,2c,2d] 2) covalently-bound oligomers through cross-linking at Tyr,[2b,2e] 3) a covalently-bound dimer of E22P-Aβ through cross-linking at Ala^{30},[2f] and 4) macrocyclic derivatives composed of Aβ fragment peptides incorporating an *N*-methylated backbone amide.[2g] To the best of our knowledge, however, there is no stable Aβ oligomer mimic that is switchable into a fibril state. Because the oligomer state of Aβ naturally transforms into the fibril state, conferring the biomimetic, aggregation-state switchable characteristics to an engineered oligomer mimic might be of significant outcome. Such system offers unexplored insight into the roles of Aβ oligomers and fibrils by temporally dissecting the dynamic process of Aβ aggregation. Here, we developed a stable Aβ oligomer mimic that can be converted into fibrils by a chemical stimulus.

Results and discussion

We focused on the most pathologically important isoform, Aβ42.[3] A salt-bridge between the side-chains of Asp^{23} (carboxylate) and Lys^{28} (ammonium) in Aβ42 is key to forming a β-strand-loop-β-strand (i.e., β-hairpin) structure, which is a fundamental motif for generating the toxic oligomer aggregates of Aβ (**1**, Fig. 1).[2d,4] Recent analyses revealed that the Asp^{23}/Lys^{28} salt-bridge is not present in the fibril state of Aβ42, allowing a new salt-bridge to form between the Lys^{28} side-chain and the Ala^{42} C-terminus[5] or Asp^{1} side-chain.[6,7] Accordingly, we designed **2** as a unique Aβ42 mimic containing an oxime functionality as a surrogate for the Asp^{23}/Lys^{28} salt-bridge of Aβ42 oligomers. Because oxime bond-formation/cleavage is reversible,[11] the aggregation state of the

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mimic (i.e., oligomer/fibril exchange) might be switchable via closing/opening of the oxime linker. A sulfoxide isoform at

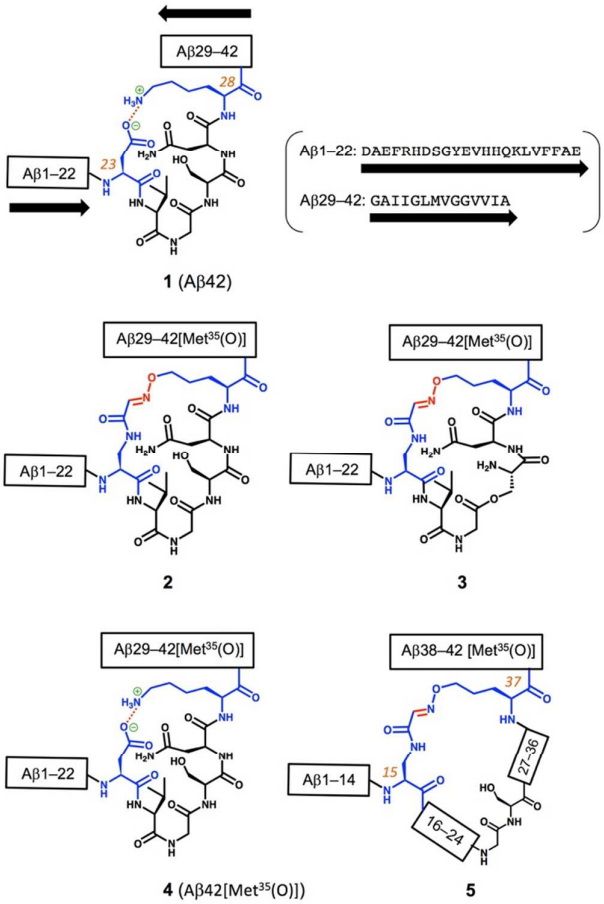


Fig. 1. Simplified chemical structures of Aβ and their mimics.

Met^{35} (Met^{35}(O)) was adopted to minimize excessive aggregation,[12] conferring favorable handling properties.[13]

We utilized *O*-acyl isopeptide **3** as a precursor of **2** in our biochemical analysis (Fig. 1). In **3**, the amide bond of **2** between Gly^{25}–Ser^{26} was isomerized into an ester bond at the hydroxy group of Ser^{26}. The *O*-acyl isopeptide of Aβ42 markedly increased the water-solubility and attenuated its aggregation propensity, allowing for easy handling during the synthesis and biochemical evaluations.[14] Under physiologic conditions at neutral pH, the *O*-acyl isopeptide was converted to Aβ42 by a facile *O*-to-*N* acyl rearrangement at Gly^{25}–Ser^{26} (Fig. S1-S3).

The 42-mer peptide chain of **3** including a single ester bond was constructed using a Fmoc-based solid phase peptide synthesis; Fmoc-Dap[Boc-Ser(*t*Bu)]-OH (Dap, 2,3-diaminopropionic acid) and Fmoc-Gly($CH_2CH_2CH_2ONHBoc$)-OH were incorporated at the 23rd and 28th positions, respectively. Following the peptide chain elongation, the resulting peptide-resin was treated with a trifluoroacetic acid (TFA)-cocktail to remove the protecting groups on the side-chains and disconnect the peptide from the resin. After rough purification of the resulting peptide, the amino alcohol component at the Ser residue connected with the Dap^{23} residue was oxidatively cleaved to the corresponding aldehyde by treatment with sodium periodate ($NaIO_4$).[11c,15] Intramolecular oxime bond-formation spontaneously proceeded between the aldehyde and hydroxyamine moieties at the 23rd and 28th positions, respectively, affording the desired isopeptide **3** (Fig. S2).

Isopeptide **3** was dissolved in 0.1% aqueous TFA, and the solution was ultra-centrifuged to remove any possible aggregates that accumulated during the synthesis and purification. Thus, we obtained a stock solution of **3** (200 μM). While **3** was stable in the acidic stock solution (0.1% aqueous TFA), when the stock solution was diluted with phosphate buffer (pH 7.4) **3** was mostly converted to **2** within 10 min at room temperature via the *O*-to-*N* acyl rearrangement reaction (Fig. S3).

We used atomic force microscopy (AFM) to examine the morphologic behavior of **2** (Fig. 2a). When a stock solution of **3** was diluted with a neutral buffer (0.1 M HEPES, 0.1 M NaCl) and incubated for 10 min, we observed a small, globular species with a z-height of 2 to 4 nm derived from **2**, which were morphologically analogous to Aβ oligomers[16] (Fig. 2a, left panel and Fig. S4). When **3** was directly analyzed by AFM by diluting a stock solution of **3** with 0.1% aqueous TFA instead of the neutral buffer (i.e., without *O*-to-*N* acyl rearrangement), however, we observed a much lower number of particles and the observed z-height was less than 1 nm (Fig. S5). This result suggests that *in situ*-generated **2** promptly formed oligomers. The oligomers were mostly preserved throughout the 24-h incubation (Fig. 2a). In sharp contrast, Aβ42 containing a Met^{35} sulfoxide without the tether (Aβ42[Met^{35}(O)]: **4**)[12] formed few visible oligomers after 3-h incubation, but robust amyloid fibrils were observed after 24-h incubation (Fig. 2b). Moreover, when the oxime linker was introduced at an alternative position between residues 15 and 37 (**5**, Fig. 1), markedly fewer oligomers formed (Fig. S6a, b). Thus, oxime linkage between the side-chains of residues 23 and

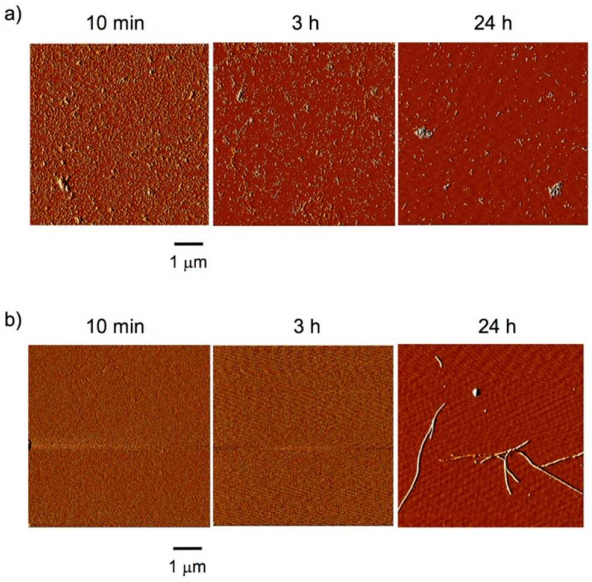


Fig. 2. AFM images of (a) **2** (10 μM) and (b) **4** (10 μM). All samples were incubated in 0.1 M HEPES buffer containing 0.1 M NaCl (pH 7.4) at 37 ˚C for the indicated time periods and then analyzed. Scale bars measure 1 μm. The images are shown in amplitude mode.

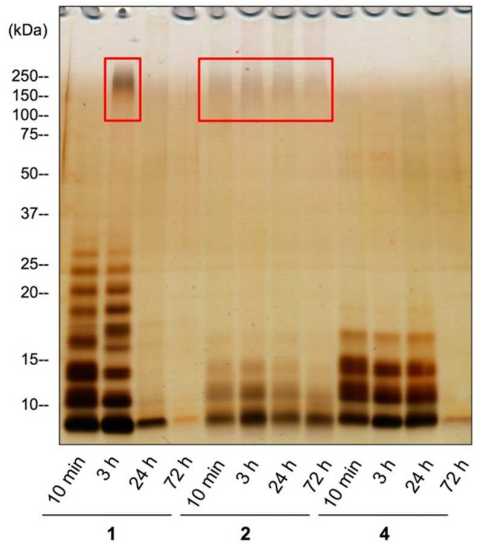


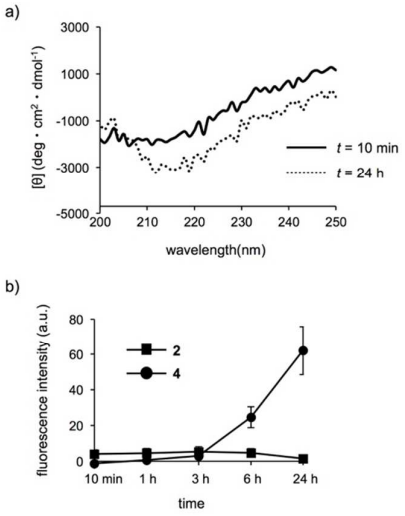
Fig. 3. SDS–PAGE analysis of **1** (20 μM), **2** (20 μM), and **4** (20 μM) after PICUP. Samples were incubated in phosphate buffer (10 mM, pH 7.4) at 37 ˚C for the indicated time-periods and then analyzed. The aggregates were separated using a 15% Tris-glycine gel and detected with silver staining.

28 of **2** was critical to form stable oligomers.

Differences in the aggregation propensities between **2**, **4**, and **5** were also observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis after photo-induced cross-linking of unmodified proteins (PICUP).[17] Thus, phosphate buffer solutions of **2**, **4**, and **5** were incubated for various lengths of time, and subjected to the cross-linking treatment using tris(bipyridyl)Ru(II) and ammonium persulfate under visible light irradiation. The oligomer distributions were analyzed by SDS-PAGE on 15% Tris-glycine gels with silver staining detection (Fig. 3). In the case of **2**, a smear band around 150–250 kDa, corresponding to large oligomers, was detected at *t* = 10 min, in addition to small oligomers (dimer, trimer, and tetramer). The large oligomer band was retained throughout the incubation time of *t* = 72 h. In the case of **4**, bands derived from the monomer and small oligomers were detected at *t* = 10 min. The oligomer distribution pattern was retained at *t* = 24 h, but the bands were hardly detectable at *t* = 72 h, presumably due to the formation of large aggregates (i.e., fibrils) unable to enter the gel (Fig. 3). The large 150~250-kDa oligomer band of **4** never appeared throughout the time-course. In the case of **5**, very few bands corresponding to oligomers were detected at any time-point (Fig. S6c). These results are consistent with the AFM analysis shown in Fig. 2 and S6ab.[18,19]

It is worth noting that a similar 150~250 kDa oligomer band appeared when using wild-type Aβ42 (**1**, *NOT sulfoxide form*, *t* = 3 h, Fig. 3). The oligomer band of wild-type Aβ42 was, however, transient and diminished as further aggregation proceeded over time (*t* = 24 h and 72 h, Fig. 3). Thus, the results of the AFM and SDS-PAGE analyses indicate that **2** readily formed fairly stable large oligomers.

Fig. 4. A phosphate buffer (pH 7.4) that contained **2** (10 μM) or **4** (10 μM) was incubated at 37 ˚C for the indicated time periods. (a) CD spectrum of **2**; (b) ThT fluorescence assay of **2** and **4** (n = 3, mean ± s.d.).



We next assessed the higher-order structure of **2** by circular dichroism (CD) spectroscopy. The CD spectrum of **2** in a phosphate buffer solution indicated the presence of a significant amount of β-sheet structures at *t* = 10 min (Fig. 4a). After 24 h, the β-sheet content was predominant, as the negative maximum at 218 nm, typically attributed to β-sheet structures, was more pronounced. This result supports the prompt formation of the β-sheet-rich structure from **2**. Compound **4**, however, existed in a random coil structure by *t* = 6 h, and the β-sheet structure was formed after a considerable incubation time (*t* = 24 h) (Fig. S7).

The fluorescence intensity of thioflavin-T (ThT) dye, which corresponds to the extent of the cross-β-sheet structure, a common quaternary structure in amyloid fibrils,[20] remained at a low level (~4 a.u.) throughout the incubation time in the case of **2** (t = 10 min, 1 h, 3 h, 6 h, and 24 h, Fig. 4b). These results are consistent with previous reports;[21] the ThT fluorescence intensity of the oligomers was considerably weaker than that of the protofibrils and matured fibrils due to the undeveloped cross-β-sheet structure in the oligomer state. Compound **4**, however, had a markedly different time-dependency. Although no fluorescence was observed at t = 10 min and 1 h, the intensity began to increase at t = 3 h (~3 a.u.), followed by a substantial increase at t = 6 h (24 a.u.) and 24 h (62 a.u.). This result indicates that **4** formed extensive amyloid fibrils composed of cross-β-sheets after a lag phase (nucleation phase).

Next, to examine the cytotoxicity of **2**, we applied **2** to rat pheochromocytoma PC12 cells at concentrations up to 10 μM. The cell culture was then incubated and cell viability was examined after a 3-day incubation (Fig. S8). Treatment with **2** reduced the viability of the PC12 cells in a dose-dependent manner. The cytotoxicity of **2** was similar to that of **4**. Thus, the potent cytotoxic characteristics of the amyloid aggregates were preserved in **2**.

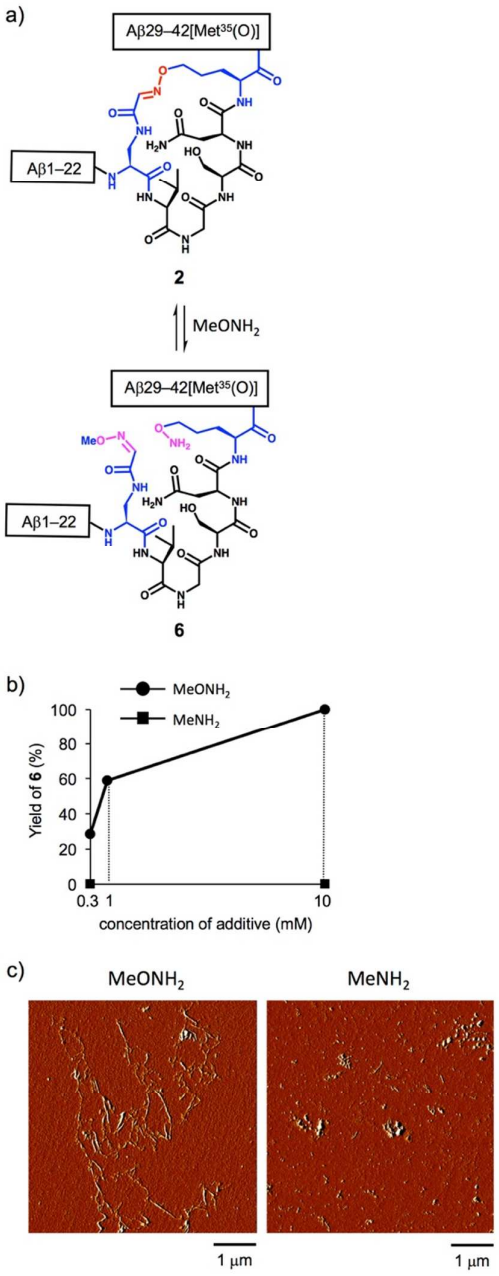


Fig. 5. Closing/opening of the oxime tether as a switch for the aggregation state of the Aβ mimic **2**. (a) Equilibrium between **2** and **6** in the presence and absence of $MeONH_2$; (b) Yield of **6** from **2** (0.1 mM) in the presence of $MeONH_2$ (0.3, 1.0, and 10 mM) or $MeNH_2$ (0.3 and 10 mM); (c) AFM images of **2** (0.1 mM) in the presence of 10 mM $MeONH_2$ where **6** is dominant or $MeNH_2$ where **2** is dominant. The sample was incubated in 0.1 M HEPES buffer containing 0.1 M NaCl at 37 °C. Scale bars measure 1 μm. The images are shown in amplitude mode.

As mentioned above, we designed an oxime tether to take advantage of the reversibility of oxime formation and cleavage.[11] Thus, we attempted to convert the stable large oligomers of **2** into fibrils by cleaving the oxime functionality as a potential aggregate state switch (Fig. 5a). After the formation of large oligomers from **2** by incubating **2** (0.1 mM) in a phosphate buffer solution (pH 7.4) for 1 h, $MeONH_2$•HCl (0.3 mM) was added. As a result, approximately 30% of the open form **6** relative to **2** was detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry at t = 24 h (Fig. 5b and S9a). In addition, the yield of **6** increased with an increase in the stoichiometry of $MeONH_2$•HCl; 60% and 99% of **6** was detected in the presence of 1 mM and 10 mM of $MeONH_2$•HCl, respectively (Fig. 5b and S9bc). When $MeNH_2$•HCl was used instead of $MeONH_2$•HCl at a concentration of 10 mM, however, **2** remained intact (Fig. 5b and S10).

We then examined the morphologic change induced by cleavage of the oxime tether of **2**. $MeONH_2$•HCl (10 mM) was added to a buffer solution containing **2** (pH 7.4, 0.1 M HEPES, 0.1 M NaCl, 0.1 mM **2**) to afford a solution in which **6** was dominant. After incubating the reaction mixture for 24 h, we analyzed the solution using AFM. As a result, fibrils were observed (Fig. 5c, left panel). When $MeNH_2$•HCl was used instead of $MeONH_2$•HCl under otherwise identical conditions, the obtained AFM image was similar to that after 24-h incubation of **2** (Fig. 2a, right panel); no formation of the fibrils was observed and the observed z-height of the particles was 2 to 4 nm (Fig. 5c, right panel, S11). Thus, cleavage of the salt-bridge surrogate tether of **2** induced the aggregation state change from oligomers to fibrils.[22] Comparative toxicity study of the oligomer formed from compound **2** and fibril formed from compound **6** indicated that both aggregate states possessed similar extent of toxicity at 10 μM (Fig. S13).

Conclusions

We report the first chemically-engineered Aβ mimic **2**, whose dominant aggregate state is switchable from oligomers to fibrils. Compound **2** contained an oxime tether as a surrogate for the salt-bridge between residues 23 and 28 in Aβ42 oligomer. Stable, large oligomers were rapidly and homogeneously formed from **2**, and the amyloid-like properties, such as the β-sheet-rich structure and toxicity, were preserved in the aggregate state. Comparable investigations between compound **2** and compound **4**

(Aβ42[Met^{35}(O)] without oxime structure) clearly suggest that the oxime tether is a requisite for the preferential adoption of oligomer state. In addition, because a corresponding Aβ42 derivative containing a lactam tether at Asp^{23}/Lys^{28} side chains also preferred the oligomer formation,[7] covalently bound tether at Asp^{23}/Lys^{28} side chains, rather than the oxime structure itself, largely attributed to the oligomer formation of **2**. Moreover, chemoselective cleavage of the oxime tether led to the aggregate state changing from oligomers to fibrils. Thus, closing/opening of a single oxime linker induced considerable impact on the aggregation-state of Aβ. Due to the transient characteristics of amyloid protein oligomers, chemical protein engineering-based methods to stabilize the toxic aggregate form should provide valuable information about structure-toxicity relationships. This novel system enables temporal dissection of the dynamic process of Aβ aggregation, which might offer unexplored insight into the roles of Aβ oligomers and fibrils.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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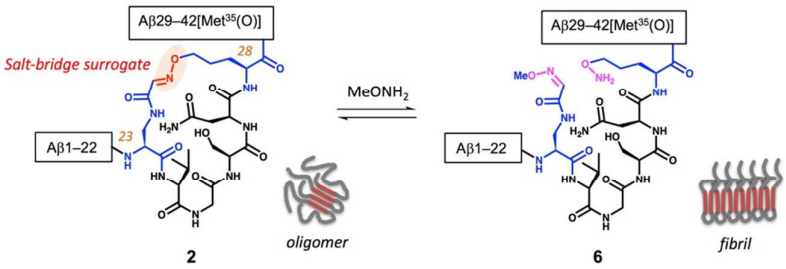
22 When $MeONH_2$ was removed via ultrafiltration for 2 h, re-conversion of the open form **6** to the closed form **2** was observed, affording a mixture of **2** and **6** in an approximately 2:1 ratio (Fig. S12).

TOC



A stable Aβ oligomer mimic that is transformed into fibrils by a chemical stimulus, i.e., an oxime exchange reaction, is disclosed.