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Hetero-assembly of a dual β-amyloid variant peptide system

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Self-assembly of amyloid polypeptides (1) imparts biological effects depending on size in over 20 amyloid diseases and (2) produces useful yet relatively untapped biomaterials. Unfortunately, our understanding of amyloid polypeptides, as related to biomedical implications and biomaterial applications, are limited by their self-assembled nature. In this study, we report creation of a dual peptide system, where a pair of β-amyloid (Aβ) variants are not self-assembled but hetero-assembled in the presence of their assembly partners. We provide evidence that the resulting hetero-assemblies share molecular, structural and morphological similarities with typical amyloid self-assemblies formed by a single polypeptide (e.g., Aβ). We anticipate that our dual peptide system may readily be adapted for precise control of amyloid assembly, for the study of size-dependent neurotoxicity and precise fabrication of amyloid biomaterials.

Self-assembly of peptides represents a central molecular event responsible for pathology of many amyloid diseases, as shown with β-amyloid (Aβ) implicated in Alzheimer's disease (AD).[1, 2] The molecular event is heterogeneous, producing a mixture of assemblies. In general, structurally disordered amyloid monomers spontaneously self-assemble to β-sheet rich oligomeric assemblies, which aggregate further to form fibrils of highly ordered cross β-sheet structure.[3-7] Amyloid assemblies can be toxic, for example, by disturbing cell membranes or causing oxidative stress.[1, 2] Despite its scientific and biomedical importance, biological manifestations of amyloid assembly still remain elusive and often contradictory,[8-10] in part due to the heterogeneous nature of the supramolecular assembly and lack of control thereof. For example, structural characterizations at high resolution and in-depth biological profiling of toxic amyloid assemblies still remain a challenge.[9, 10] The limitation originates from the self-assembled nature of amyloid peptides, which associate spontaneously. A similar limitation is realized during fabrication of amyloid-based biomaterials, particularly in fibrillar forms,[4-6] which are advantageous due to their superior chemical, thermal, and mechanical stability for creation of nanowires, hydrogels, and catalytic materials.[11, 12] Amyloid aggregation often involves multiple amyloid polypeptides. For example, amyloid fibrils can cross-seed assembly of other amyloid proteins.[13] Amyloid fibrils formed by a hydrolysed globular protein often comprise its multiple peptide fragments.[14]

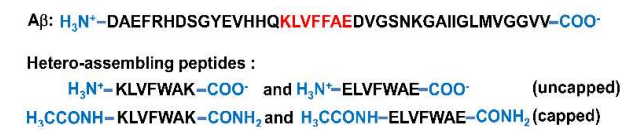

Figure 1. Amino acid sequences of Aβ and hetero-assembling peptides. The Aβ fragment from which the hetero-assembling peptides were derived is shown in red. The terminal charges and their modifications are

In this paper, we report engineering of a hetero-assembly system, where a pair of Aβ variants, KLVFWAK and ELVFWAE (**Fig. 1**), do not self-assemble, but instead form amyloid assemblies upon mixing. In this system, growth of amyloid assemblies is arrested until addition of hetero-assembly partners. Due to such nature of hetero-assembly, our dual peptide system may readily be adapted for precise control of amyloid assembly (**Fig. S1**), which has long been recognized as challenging with an amyloid peptide.[9, 15, 16] Toxicity of amyloid assemblies greatly depends on their size.[17, 18] Evidence has suggested that maximal toxicity may be realized with intermediate sized assemblies.[19, 20] Unfortunately, a consensus on the specific "toxic" size has not yet been established,[21] in part due to difficultly in purification of size-specific subpopulations of amyloid assemblies necessary to determine size-dependent toxicity.[19, 21] While cross-linking followed by size-based purification could produce size-specific amyloid subpopulations, this method seemed inefficient for preparation of intermediate to large size aggregates.[22] We envision that our system can be exploited for controlled amyloid growth (**Fig. S1**), a novel tool for the exploration of size-toxicity relationships in neurodegenerative diseases. A similar hetero-assembly approach can also lead to the fabrication of amyloid-based biomaterials with controllable compositions of functional domains (**Fig. S1**).

Recently, hetero-assembly of *de novo* peptides, composed of 11 alternating hydrophilic and hydrophobic residues, and their fusion to a globular protein have been reported.[23] While the study proved that functional amyloid fibrils can be produced

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through spontaneous hetero-assembly, the molecular basis of hetero-assembly as it relates to amyloid diseases – in terms of oligomer formation, fibril structure, molecular arrangement, and peptide binding affinity – has yet to be studied. It should also be noted that many pathologically relevant amyloid sequences (e.g., Aβ) do not follow such alternating primary structure. Moreover, peptide fusion to a globular protein often compromise its stability,[24] and benefits from tagging of shorter rather than longer peptides. In our study, we show evidence that the hetero-assembly from the 7-residue peptides exhibit molecular, structural and morphological similarities with typical amyloid self-assembly. Our peptides are derived from Aβ, increasing their biological relevance. We also show that the molecular properties of hetero-assemblies can be controlled by modulation of balance between hydrophobic and electrostatic interactions.

The Aβ variant pair, KLVFWAK and ELVFWAE, was derived from a hydrophobic central domain (HCD), KLVFFAE, critical in Aβ self-assembly (**Fig. 1**). Many Aβ variants have been derived from the Aβ HCD and shown to self-assemble, including KLVFFAE.[25, 26] Unlike KLVFFAE, KLVFWAK display limited homo-assembly (that is, self-assembly among same species) at neutral pH, due to the charged terminal lysine residues.[27, 28] In the present study, we created a hetero-assembly partner of KLVFWAK, ELVFWAE, via terminal point mutations, to electrostatically drive hetero-assembly while minimizing homo-assembly. Additionally, the core contains a hydrophobic LVFWA motif to stabilize β-sheet rich amyloid assemblies via hydrophobic interactions.[29] In the peptide variants, F5W mutations were included for concentration estimation based on UV absorbance. Impact of this mutation on amyloid assembly is likely to be minimal as was shown with other Aβ fragments.[12, 30]

As was the case with KLVFWAK[27], ELVFWAK also displayed limited homo-assembly during prolonged incubation in aqueous

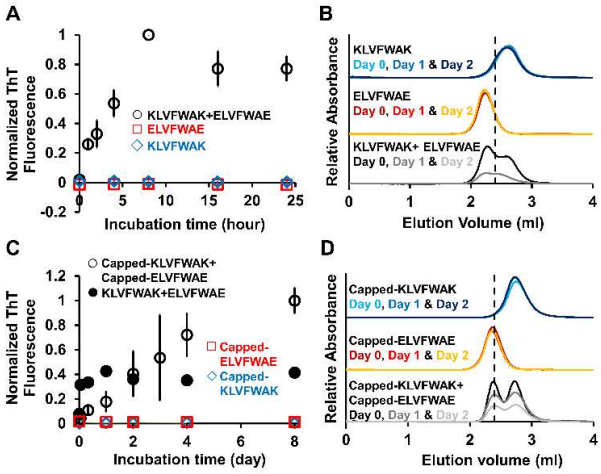

Figure 2. Aggregation of peptides (A-B) without and (C-D) with terminal capping as monitored by (A,C) ThT fluorescence and (B,D) SEC. Peptide concentration: (A-B) total 200 μM and (C-D) total 400 μM per each sample. The mixtures of KLVFWAK + ELVFWAE and capped-KLVFWAK + capped-ELVFWAE are equimolar. In (A) and (C), ThT fluorescence intensities of samples were normalized by those at the maximum of the mixtures containing uncapped and capped peptides, respectively. In (B) and (D), the vertical dotted lines indicate column volume.

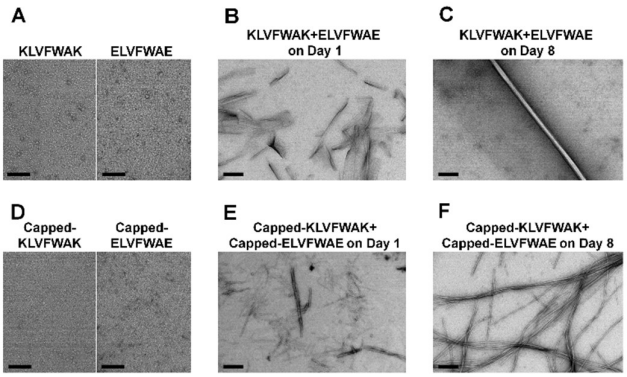

Figure 3. TEM images of peptides (A-C) without and (D-F) with terminal capping. Scale bars: 100 nm in (A) and (D), and 200 nm in (B-C) and (E-F)

buffer, as judged by laser light scattering (LLS) (**Table S1**), fluorescence of Thioflavin T (ThT), a dye emitting signal upon recognition of amyloid self-assemblies rich in β-sheet structure,[31] (**Fig. 2A**) and size exclusion chromatography (SEC; **Fig. 2B**). On SEC, elution volumes of KLVFWAK and ELVFWAE were larger than that of aprotinin (6.5 kDa), consistent with the molecular weight of each peptide (~1 kDa). Individual peptides of KLVFWAK and ELVFWAE eluted immediately after and before the column volume, respectively. We interpreted this finding as a result of interactions between peptides and column resins due to charged residues. Limited homo-assembly was confirmed with no change on SEC peaks during incubation (**Fig. 2B**) and transmission electron microscopy (TEM; **Fig. 3A**). Similar to KLVFWAK, ELVFWAE remained structurally disordered when incubated alone, as determined by circular dichroism (CD; **Fig. 4A**). The spectra of KLVFWAK or ELVFWAE resembled that of structurally disordered Aβ monomers,[32] indicating the relevance of our system to Aβ.

Mixing KLVFWAK and ELVFWAE at 100 μM each with 150 mM NaCl resulted in rapid hetero-asembly (**Table S1**). During incubation, the mixture exhibited a significant increase in ThT fluorescence similar to typical amyloid aggregation,[33] but unlike the individual peptides (**Fig. 2A**). Also, as seen with Aβ self-assembly, hetero-assembly kinetics is concentration-dependent: maximum ThT signals were observed after ~8 hour and ~1 hour at 200 and 400 μM total peptide concentrations, respectively (**Fig. 2A, C**). Similarly, precipitation occurred faster with mixtures at higher peptide concentrations (**Table S2**). When the soluble fractions of peptide mixtures were examined by SEC, peaks other than those observed for unmixed samples were not detected (**Fig. 2B**). The size exclusion chromatogram of the soluble fractions of peptide mixtures indicates that KLVFWAK and ELVFWAE were incorporated into precipitates at ~1:1 ratio (**Fig. 2B** and **Table S3**), which was confirmed with fluorescently-labeled peptides (**Supporting Text**).

A TEM analysis revealed that the hetero-assemblies formed at 200 μM total peptide concentration appeared as globular aggregates ~10 nm in diameter during the early stage of incubation (3 hour; **Fig. S2**). Soluble globular oligomers of a similar size were previously detected during Aβ self-assembly[4, 34]. The lack of SEC peaks indicative of the globular oligomers (**Fig. 2B**) may result from their dissociation due to dilution

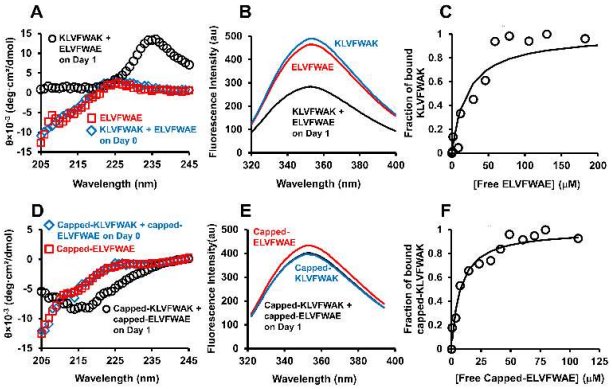

Figure 4. Structural changes upon equimolar mixing of (A,B) KLVFWAK + ELVFWAE, and (D,E) capped-KLVFWAK + capped-ELVFWAE, as determined by (A,D) CD spectroscopy and (B,E) tryptophan fluorescence. (C,F) The fraction of KLVFWAK bound to ELVFWAE as determined by tryptophan fluorescence quenching when termini of the peptides are (C) uncapped or (F) capped.

effects during SEC. Further incubation of the mixture led to emergence of flat nanotapes with a width of ~40-60 nm (**Fig. 3B**). The mixture then formed straight mature fibrils upon prolonged incubation (**Fig. 3C**). The fibrillar species appeared a few μm in length and 10–35 nm in width, similar to other amyloid fibrils.[6] The polymorphic nature (i.e., nanotapes vs. fibrils) may depend on mesoscopic arrangements of aggregates (e.g., packing and twisting) and their energetics. [35] The transient flat amyloid morphology might be related to an amyloid polymorph, amyloid crystal, which was proposed to occupy the ground state in energy landscape, though conversion of amyloid crystals to fibrils seems energetically unfavourable.[36] Decreasing total peptide concentration to 150 μM resulted in relatively short protofibrils and globular aggregates (**Fig. S3**), confirming the concentration-dependency.

Structural changes of the peptides occurred during hetero-assembly as characterized by an increase in positive ellipticity at ~235 nm in CD (**Fig. 4A**), indicative of exciton coupling of neighboring tryptophan residues at distances of ≤~10Å.[37] Consistent with the presence of tryptophans in close proximity,[38] we observed tryptophan fluorescence quenching of 40% (**Fig. 4B**) when an equimolar peptide mixture was incubated for 1 day at 150 μM total where the peptides remained soluble, indicating that the quenching is not due to reduction of soluble peptide concentrations. Tryptophan fluorescence quenching of 25 μM KLVFWAK upon addition of 0-200 μM ELVFWAE was also monitored (**Fig. 4C**). With the assumption of 1:1 binding between the peptides, apparent binding affinity K_D was evaluated to be 20 μM, which is in the range of K_D for self-assembly of Aβ and its fragments.[39, 40]

Attenuated total reflectance (ATR)-FTIR was used to determine secondary structure of fibrillar hetero-assemblies (**Fig. 5A**). Deconvolution of the spectra revealed that the resulting structures were rich in β-sheets (**Table S4**). The presence of peaks at 1625 and 1695 cm^{-1} is indicative of anti-parallel orientation.[41] X-ray diffraction (XRD) revealed fibril *d*-spacing values of ~4.7 and ~10 Å (**Fig. 5B)**, similar to the spacing of the multi-layer cross β-sheets found in Aβ fibrils.[28, 29]

Based on our peptide design, we reasoned that a proper balance between electrostatic and hydrophobic interactions is important for hetero-assembly, which was also experimentally confirmed (see **Supporting text** and **Fig. S4-S6**). Thus, we utilized terminal capping to isolate the electrostatic interactions to the side chains only (**Fig. 1**). The resulting peptides are referred to as capped-KLVFWAK and capped-ELVFWAE. Like uncapped peptides, unmixed capped peptides displayed limited homo-assembly (**Table S5**, **Figs. 2C-D** and **Fig. 3D**), and were found to be structurally disordered (**Fig. 4D**).

Mixing the equimolar capped peptides at 400 μM total initiated hetero-assembly (**Table S5** and **Fig. 2C**). Notably, terminal capping of the mixture reduced the rate of ThT fluorescence increase substantially: unlike uncapped peptides, no ThT fluorescence change was observed after 1 day of incubation when the total capped peptide concentration was 200 μM (data not shown). The result is consistent with the aforementioned view that hetero-assembly depends on a balance between electrostatic and hydrophobic interactions. Nearly equimolar incorporation of capped-KLVFWAK and capped-ELVFWAK into hetero-assemblies was confirmed (**Fig. 2D** and **Table S3**). The sample also produced amyloid fibrils (**Figs. 3E-F**), which were narrower when compared to those formed by uncapped peptides. The reduced fibril width may be caused by limited lateral association of peptides due to terminal charge neutralization.

After incubation for 1 day, the CD spectrum for the equimolar mixture with capped peptides exhibited a minimum at ~218 nm (**Fig. 4D**), indicative of β-sheet secondary structure. Unlike uncapped peptide mixtures, no positive ellipticity at 235 nm was detected in the CD spectrum (**Fig. 4D**), indicating tryptophans are further apart in capped hetero-assemblies. Consistent with this finding, the degree of fluorescence quenching during hetero-assembly was lowered with capped peptides (6 vs. 40%) (**Fig. 4E** vs. **4B**). K_D for capped peptides was estimated to be ~7 μM, which is in a similar range for uncapped peptides, as determined by fitting tryptophan fluorescence quenching data, assuming 1:1 binding (**Fig. 4F**). Mixing capped peptides also produced fibrils containing multiple layers of anti-parallel β-sheets (**Fig. 5A, 5C** and **Table S4**).

In conclusion, our results demonstrate that structurally disordered peptides adopt β-sheet structures upon hetero-assembly, similar to typical amyloid self-assembly. One advantage of our system over other hetero-assembling peptides arises from tryptophan exciton as a readout to provide

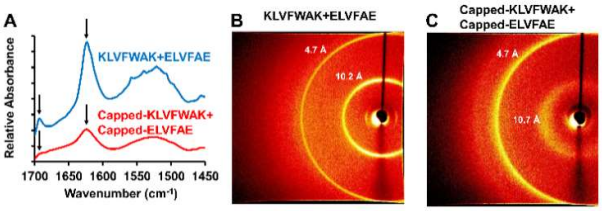

Figure 5. (A) FT-IR spectra and (B-C) X-ray diffraction patterns of fibrils formed by hetero-assembly of peptides. In (A), peaks characteristics of anti-parallel β-sheets are indicated by arrows. In (B) and (C), the dominant reflections at ~4.7 Å on the median and ~10.5 Å on the equator are shown.

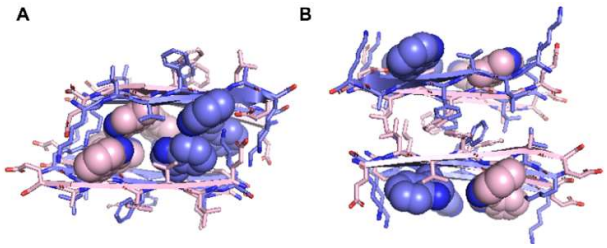

Figure 6. Structural models of hetero-assemblies (A) between KLVFWAK (blue ribbon) and ELVFWAE (purple ribbon) and (B) between capped-KLVFWAK (blue ribbon) and capped-ELVFWAE (purple ribbon) viewed from the side. Tryptophans are shown as spheres.

insight into molecular arrangement of amyloid assemblies, which was subject to a minor chemical modification (e.g., terminal capping). According to the CD, tryptophan fluorescence, ATR-FTIR and XRD data, we propose structural models of hetero-assemblies, where anti-parallel β-strands are aligned on a β-sheet with relative distance among tryptophans (or a local density of tryptophans) altered by terminal capping (**Figs. 6, S7** and **Supporting Text**). Though exact locations of residues remain unknown and other structural models may not be ruled out, it became obvious that peptide orientation on β-sheets could be directly influenced by terminal capping. We anticipate that the hetero-assembly system can readily be adapted for precise control of amyloid growth through step-wise peptide addition (**Fig. S1**).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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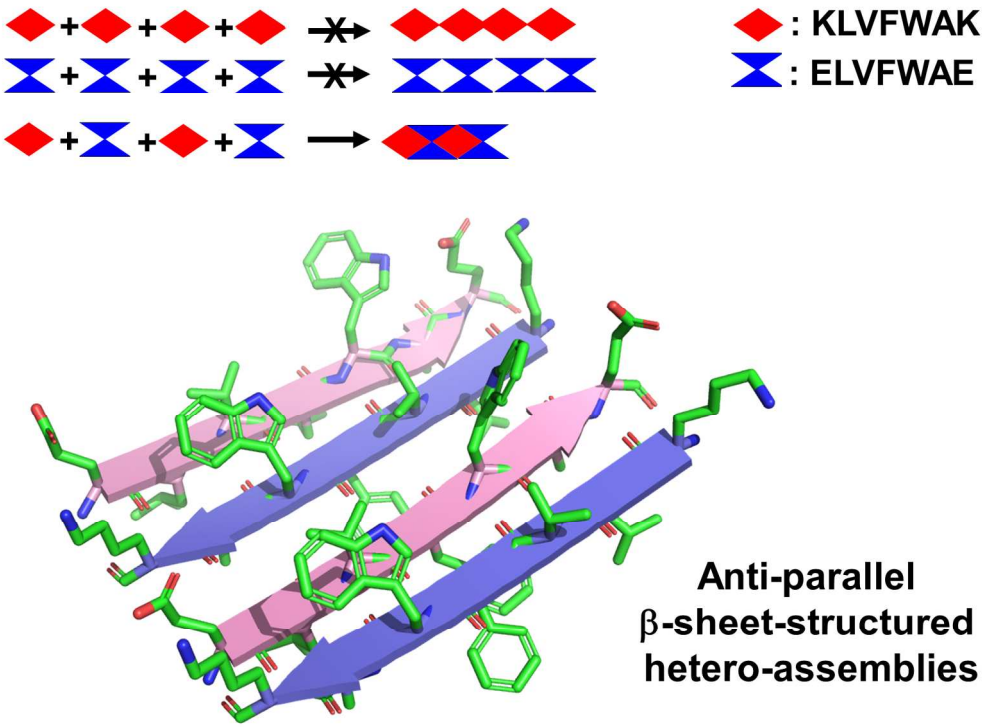
Creation of a dual peptide system where beta-amyloid variants hetero-assemble but do not homo-assemble, sharing similarities with typical amyloid self-assemblies.



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