

Chemical Probes That Selectively Recognize the Earliest A β Oligomers in Complex Mixtures

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Abstract: Alzheimer's disease (AD) is characterized by the self-assembly of amyloid beta (A β) peptides. Recent models implicate some of the earliest A β oligomers, such as trimers and tetramers, in disease. However, the roles of these structures remain uncertain, in part, because selective probes of their formation are not available. Toward that goal, we generated bivalent versions of the known A β ligand, the pentapeptide KLVFF. We found that compounds containing sufficiently long linkers (~19 to 24 Å) recognized primarily A β trimers and tetramers, with little binding to either monomer or higher order structures. These compounds might be useful probes for early A β oligomers.

Both *in vitro* and *in vivo*, the amyloid beta (A β) monomer will self-assemble into higher order structures, including dimers, trimers, tetramers, and larger oligomers. Eventually, this peptide will form the elongated fibrils that are observed in late-stage Alzheimer's disease (AD) patients (Figure 1a). Recent studies have suggested that the accumulation of smaller aggregates, not the large fibrils, might better correlate with neurotoxicity.¹ For example, patient-derived A β dimers and trimers have been shown to inhibit long-term potentiation and damage synaptic plasticity.² Similarly, synthetic A β trimers and tetramers are 2-fold more toxic to cultured neurons than A β monomers, dimers, or fibrils.³ Together, these studies suggest that some of the earliest structures in the aggregation pathway might be the most diagnostic of disease.

The A β peptide is a 40 or 42 amino acid fragment of the amyloid precursor protein (APP) (Figure 1b). Portions of this peptide are thought to form β -sheets upon release from APP, and subsequent stacking of these regions appears to nucleate A β self-association.⁴ Within these sites, residues 16 through 20, KLVFF, are especially important.⁵ Specifically, this motif is thought to interact with itself in adjacent β -strands, with the phenylalanine residues forming key, repetitive intermolecular contacts.⁶ The interactions of KLVFF with itself has been studied extensively, and these studies have suggested that many A β structures have an exposed KLVFF motif at each "end".⁷ Consistent with this model, KLVFF-based peptides will inhibit A β aggregation at high concentrations, presumably by blocking these sites.^{6c} However, KLVFF binds only weakly ($K_d > 1$ mM)⁸ and multivalent displays have been found to be required for potent inhibition.⁹ Multivalent binding is known to significantly enhance avidity and selectivity in many systems, by elevating the local ligand concentration, favoring multisite binding and other

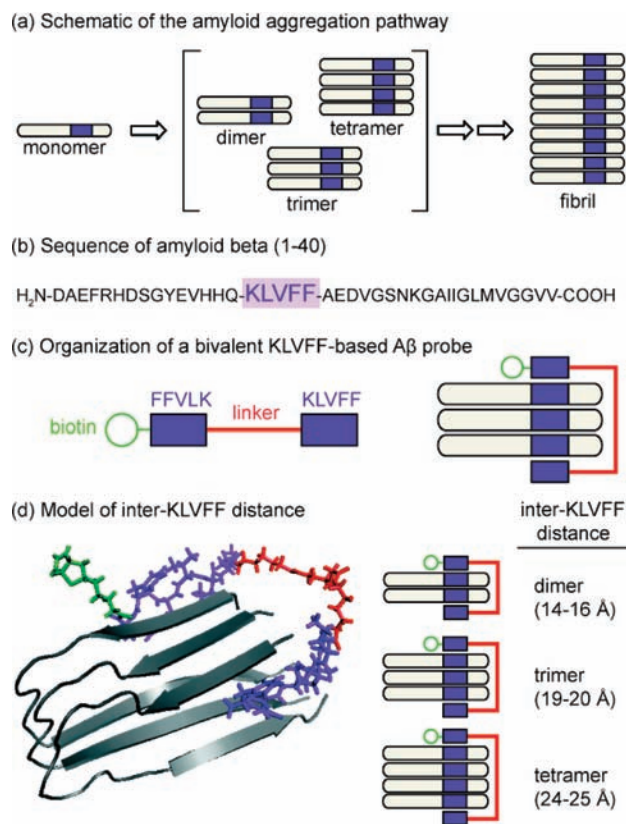


Figure 1. Design of KLVFF-based probes. (a) Schematic of the A β aggregation pathway, highlighting the earliest structures. (b) The sequence of A β (1–40), including the core KLVFF motif. (c) Proposed features of a bivalent, KLVFF-based probe. The A β is shown in gray, the KLVFF in purple, the linker in red, and the biotin tag in green. (d) Snapshot from a molecular dynamics simulation of a KLVFF-based probe bound to a model A β trimer. Based on these simulations, an approximate distance between KLVFF sites was estimated.

mechanisms.¹⁰ Based on these observations, we envisioned that bridging two KLVFF peptides with a linker of the appropriate length might provide a probe for the earliest A β oligomers (Figure 1c). This strategy was designed to address a central challenge in building probes that are specific for a subset of A β structures. Namely, these oligomers are assembled from identical monomer units, and therefore, they contain many degenerate molecular features, such as high beta-sheet content. By exploiting one of their few distinguishing properties (e.g., end-to-end distance between KLVFF motifs), we hoped to circumvent these issues.

To estimate the minimal distance needed to span the ends of an early A β aggregate, we assembled a representative KLVFF-based probe *in silico* and then employed molecular dynamics (MD)

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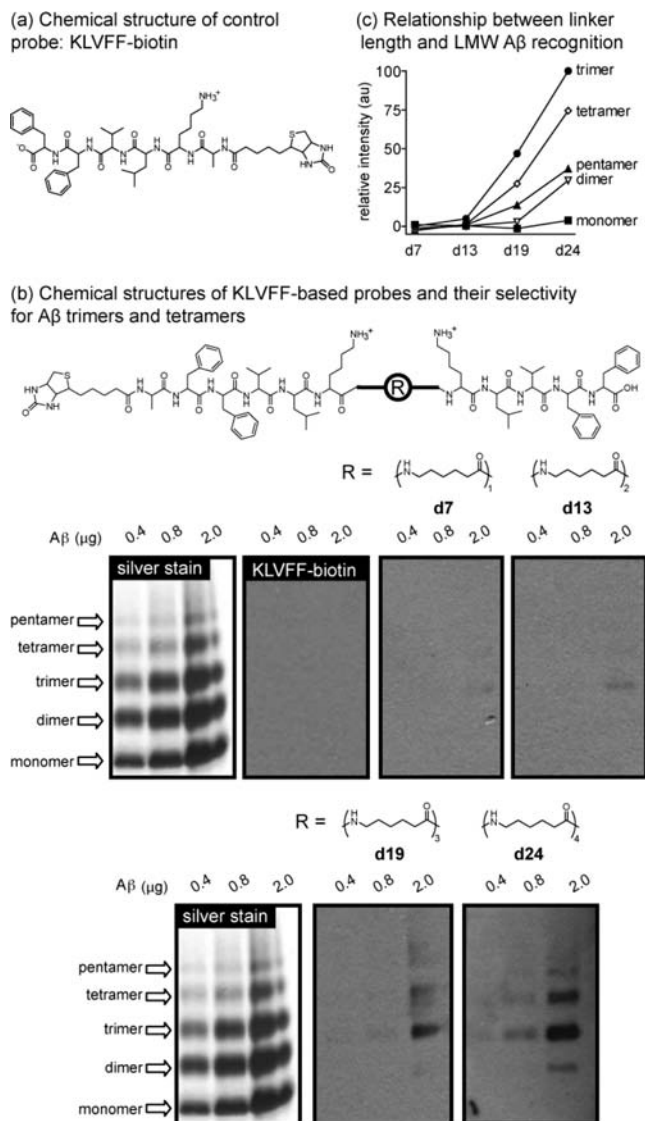


Figure 2. KLVFF-based probes selectively bind A β trimers and tetramers. (a) Chemical structure of the monovalent, KLVFF-biotin probe. (b) Chemical structures and binding properties of bivalent probes. A β (1–40) was cross-linked as described, separated using electrophoresis, and transferred to nitrocellulose. Compounds (2 μ M) were incubated with the membranes, which were then washed and imaged with streptavidin-HRP. (c) The A β band intensities are plotted against the maximum linker length. Results are representative of four independent experiments.

simulations to examine its binding to a model A β repeating unit.^{7f} These studies roughly estimated the distance between KLVFF sites as 14–16 Å in a dimer, 19–20 Å in a trimer, and approximately 24–25 Å in a tetramer (Figure 1d and Supplemental Figure 1). Using microwave-assisted, solid-phase peptide coupling, we then constructed a control compound in which the KLVFF peptide was linked to biotin (Figure 2a). Similarly, we generated four molecules (**d7**, **d13**, **d19**, and **d24**), each containing two KLVFF motifs separated by a variable number of aminohexanoic acid (Ahx) units and a biotin at the N-terminus (Figure 2b). These compounds were named according to the approximate length of the extended linker (e.g., **d7** has an estimated linker length of \sim 7 Å).¹¹ We found that these probes were soluble and nonaggregating in aqueous solution at low concentrations (below 10 μ M).

To test binding of these probes, we employed an established, UV cross-linking approach to produce A β samples containing a mixture of small oligomers.¹² Briefly, 25 μ M A β (1–40) was cross-

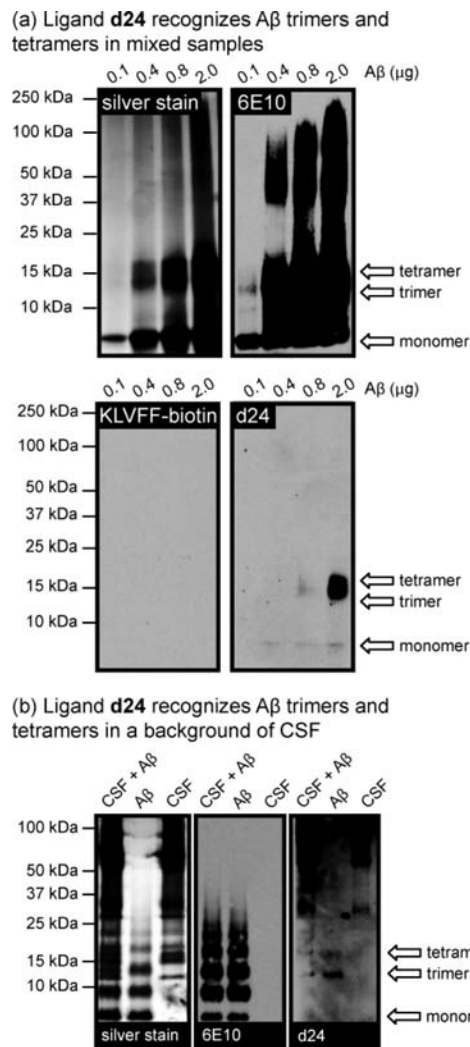


Figure 3. Ligand **d24** selectively recognizes trimers and tetramers in mixed A β samples and human cerebrospinal fluid (CSF). (a) A β (1–42) was separated and probed as described above. Silver staining or probing with the anti-A β antibody 6E10 showed a mixture of monomers, trimers, tetramers, and high-molecular weight oligomers, but **d24** bound predominantly to trimers and tetramers. (b) Cross-linked A β (1–40) was added to human CSF and probed with **d24**. Results are representative of at least two independent replicates.

linked with a Ru(II) catalyst and excess catalyst was removed.¹³ The resulting “ladder” was separated by native-gel electrophoresis. By silver staining, we observed approximately equal levels of monomer, dimer, and trimer, along with lesser quantities of tetramer and pentamer (Figure 2b). We then transferred these samples to nitrocellulose, incubated with the KLVFF probes (2 μ M) and washed extensively. The bound material was localized using streptavidin coupled to horseradish peroxidase (HRP).

Under these conditions, we observed no binding by the KLVFF-biotin control (Figure 2b), a result consistent with its weak affinity.⁸ Similarly, the KLVFF-based probes with relatively short linkers, **d7** and **d13**, also had weak binding, with a faint band at the molecular weight of an A β trimer (Figure 2b and 2c). However, the compounds with longer linkers, **d19** and **d24**, interacted strongly with the trimer and tetramer regions, with some binding to the dimers and pentamers (Figure 2b and 2c). The relatively poor binding to the A β dimers might suggest that it is not as ordered as the other structures, a concept that is consistent with recent MD and NMR studies.^{6h,7d} Importantly, the A β monomer was not

recognized by any of the ligands at these concentrations, supporting an important role for multivalent interactions.

Based upon the results using cross-linked A β samples, we wanted to further test binding in more dynamic mixtures. Toward that goal, we used ligand **d24** to probe aged samples of non-cross-linked A β (1–42). Samples prepared by this method are known to contain a mixture of monomers (4.5 kDa), poorly resolved trimers and tetramers (~12 to 18 kDa), and higher order oligomers (~40 to 200 kDa).¹⁴ Consistent with those patterns, we observed A β structures of these sizes by either silver staining or Western blots with the anti-A β antibody 6E10 (Figure 3a). The KLVFF-biotin control did not recognize any of the bands under these conditions. However, **d24** remained bound to the region corresponding to trimers and tetramers, suggesting that selectivity is maintained (Figure 3a). Minimal binding to monomers was seen, and importantly, we did not observe any interactions with the higher molecular weight oligomers, further emphasizing the selectivity of these probes for early structures in the A β aggregation pathway.

Finally, we wanted to evaluate binding in human cerebrospinal fluid (CSF), which provides a more challenging environment than aqueous buffers in which to retain binding. To determine if **d24** could still bind A β in this milieu, we added 1 μ g of cross-linked A β (1–40) to CSF samples from non-AD patients (6 μ g of total protein) and characterized the resulting mixture by silver stain, 6E10 antibody, and **d24**. From these studies, we concluded that **d24** recognized several unrelated bands within the CSF sample; however, this off-target reactivity was largely restricted to proteins >50 kDa. Thus, we were still able to visualize binding to A β trimers and tetramers (Figure 3b). Similar findings were obtained using A β (1–42) (Supplemental Figure 2). Together, these results suggest that the multivalent probes can exploit unique inter-KLVFF distances to distinguish between otherwise closely related A β structures. Based on these findings, we anticipate that derivatives of **d24**, with further improvements in affinity and selectivity, may be promising probes for detecting the appearance of the earliest A β aggregates.

Acknowledgment. A.A.R. was supported by a fellowship from the Biogerontology NIA Training Grant (AG000114). This work was also supported by a grant from the Alzheimer's Association (NIRG-08-89471) to J.E.G. The authors thank C. Evans and P. Marinec for assisting with ligand characterization, M. Wallace for helpful discussions, and D. Giachero of the UM Hospital for CSF samples.

Supporting Information Available: Detailed protocols for ligand synthesis and characterization, A β preparation and cross-linking, Western blots, and MD simulations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA106291E