Candida albicans Als Adhesins Have Conserved Amyloid-Forming Sequences

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The cell wall-bound Als adhesins of Candida albicans mediate both yeast-to-host tissue adherence and yeast aggregation. This aggregation is amyloid-like, with self-propagating secondary-structure changes, amyloid-characteristic dye binding, and increased birefringence (J. M. Rauceo, N. K. Gaur, K. G. Lee, J. E. Edwards, S. A. Klotz, and P. N. Lipke, Infect. Immun. 72:4948–4955, 2004). Therefore, we determined whether Als proteins could form amyloid fibers with properties like those in cellular aggregation. The β-aggregation predictor TANGO identified a heptapeptide sequence present in a highly conserved sequence with amyloid-forming potential in Als1p, Als3p, and Als5p. A tridecapeptide containing this sequence formed fibers that bound Congo red and thioflavin T and had characteristic amyloid morphology. Als5p20-431 and Als5p20-664, large fragments of Als5p containing the amyloid sequence, also formed amyloid-like fibers and bound Congo red under native conditions. $K_\text{d}/K_\text{f}$ analysis showed that the amyloid-forming sequences are highly conserved in Als proteins and evolve more slowly than other regions of the proteins. Therefore, amyloid-forming ability itself is conserved in these proteins.

Although amyloids are increasingly well characterized in degenerative conditions, such as Parkinson’s disease and Alzheimer’s disease, there are relatively few examples of non-pathological functions. Given that many proteins have the ability to form amyloids, this lack is somewhat surprising. Among the few known functions are the amyloid curli, adhesive appendages of gram-negative bacteria (1, 3, 32), and yeast regulatory amyloids that may mediate epigenetic diversification (31). Recently, a natural amyloid has been found to accelerate formation of ordered structures, as does amyloid formation.

The opportunistic pathogenic yeast Candida albicans has broad specificity in adhering to host tissues, and adherence is followed by biofilm formation and colonization. Among many adhesins expressed by C. albicans, the Als proteins have been implicated in pathogenesis and biofilm formation (2, 13, 23, 33). These cell wall-bound adhesins bind to diverse mammalian peptide ligands, cause cellular aggregation through homotypic adhesion (8, 16, 17), and also coaggregate with other microbial pathogens to mediate polymicrobial infections (15). Each of eight heterozygous ALS genes encodes a protein with a signal sequence, three tandem immunoglobulin (Ig)-like domains, a 127-residue Thr-rich conserved domain (T), a variable number of glycosylated 36-residue tandem repeats (TR), a highly glycosylated stalk domain, and a glycosylphosphatidylinositol addition signal sequence that mediates covalent attachment to the wall glucan (Fig. 1) (12, 29). The Ig-like domains mediate adherence, and the TR increase the affinity of the Ig-like region for ligands and can also aggregate yeasts independently of the Ig-like region (12, 20, 24, 25, 29).

Als5p is typical of the family and is sufficient to cause adherence of Saccharomyces cerevisiae to fibronectin-coated beads. The best-characterized Als5p allelic protein has 1,419 residues, including the secretion signal sequence and the glycosylphosphatidylinositol addition signal. The Ig-like regions (residues 20 to 306) and the T region (residues 307 to 433) are not glycosylated, whereas the TR (residues 434 to 649) are heavily O-glycosylated (24).

Surprisingly, yeast cell aggregation mediated by Als5p or other members of the Als family has properties that are characteristic of protein aggregation in amyloids (9, 25). Like amyloid formation, aggregation ability propagates through the adherent cell population and depends on conformational change in the Als adhesins, rather than on signal transduction and/or expression of new surface proteins. As in amyloid formation, hydrogen bonding is key, and H-bond perturbants inhibit the interaction (9, 25). Furthermore, Als5p-mediated cellular aggregates bind dyes that are used to stain amyloids, including Congo red and 8-analino-1-naphthalene-sulfonic acid (25). 8-Analino-1-naphthalene-sulfonic acid fluorescence increases greatly on binding to amyloids and on binding to yeast cells aggregated by Als5p. Additionally, aggregation induces increased cell surface birefringence (an indication of the formation of ordered structures), as does amyloid formation.

Other properties of Als proteins are also reminiscent of amyloid-like interactions. Although highly glycosylated, they have limited solubility, and the purified proteins aggregate...
from solution, even at low concentrations. Soluble Als5p and Als1p are β-sheet rich, and Als5p shows transitions between different structural states near physiological temperatures and pH values, and such structural transitions are required for amyloid formation (25). Therefore, we tested Als5p for the formation of amyloids in vitro.

MATERIALS AND METHODS

**Als5p purification.** *S. cerevisiae* W303-1B cells transformed with pRL02 or pRL09 plasmids encoding Als5p1-664 or Als5p1-431, respectively, were cultured in CSM-Ura medium with galactose as the carbon source (24). Cultures were grown for 4 days at 30°C at 175 rpm and centrifuged, and the supernatants were kept on ice. The supernatants were filtered through a 0.22-μm filter and then concentrated 10-fold using a Millipore filtration apparatus with a molecular cutoff of 30 kDa. The protein suspension was adjusted to pH 7 with Tris base. The protein was bound to a prewashed and equilibrated Ni-nitrilotriacetic acid column with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 7). Non-specific proteins and other substances were removed from the column with wash buffer, and then the protein was eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, pH 7). The eluted fractions with proteins (verified by dot blot analysis) were pooled and dialyzed exhaustively in 20 mM sodium phosphate buffer, pH 6.2, at 5°C with stirring, using a dialysis membrane with a molecular cutoff of 8,000 Da. The protein aggregated upon concentration by dialysis against 10% polyethylene glycol with an average mass of 35 kDa in 20 mM sodium phosphate buffer, pH 6.2, at 5°C, and then left unstirred for 2 days at 5°C. Protein concentrations were determined as A280, with an extinction coefficient of 1 ml/mg. We assumed that the purified proteins had lost their signal sequences, and therefore they were called Als5p20-664 and Als5p20-431, respectively.

**Dot blots and immunoblots.** Protein samples were spotted or electrophoretically transferred onto nitrocellulose membranes and allowed to dry. The membranes were rehydrated and blocked with phosphate-buffered saline (PBS) (10 mM NaH2PO4, 137 mM NaCl, 1.8 mM KH2PO4, 2.7 mM KCl, pH 7) with 5% nonfat dry milk for 1 hour. The membranes were washed three times with PBS with 0.1% Tween 20, probed with anti-V5 or anti-His, peroxidase-conjugated antibodies at a working concentration of 1:5,000 for 1 hour, and then washed three times in PBS with 0.1% Tween 20. The presence of protein was detected by incubating the membrane with equal amounts of Super Signal West Pico Stable Peroxide and Super Signal West Pico Lumino/Enhancer solutions (Pierce). Luminescence was detected on X-ray film.

**Polyacrylamide gel electrophoresis.** Als5p samples were electrophoresed on 4 to 20% precast native and nonnative gels at room temperature or in the cold. The gels were stained with Coomassie blue dye overnight at room temperature with slow shaking and then destained with 5% acetic acid solution with 10% ethanol. The molecular weights of the proteins were determined by running Bio-Rad Rainbow molecular weight markers on the same gel.

**Peptide aggregation.** The peptides SNGIVIVATTRTV and SNGINIVATTRTV were synthesized and purified to at least 95% purity by the Arizona State University peptide synthesis facility before use. Their identities and purities were checked by mass spectrometry at the Rockefeller University peptide facility. To generate amyloid, the peptides were suspended in deionized water (2 mg/ml), stirred for 2 days at 5°C, and then left unstirred for 2 days at 5°C (14). Control suspensions of SNGIVIVATTRTV were similarly incubated without stirring.

**Thioflavin T amyloid assay.** Thioflavin T (Sigma) (0.8 mg/ml in PBS) was filtered through a 0.2-μm syringe filter and used as stock solution. The solution was kept in the dark. Two 50-fold serial dilutions of thioflavin T stock solution were made, and the absorbance was determined at 440 nm. The fluorescence intensity of a 1-ml working solution was measured using a Fluorolog (Jobin Yvon-Spex) fluorescence spectrometer at an excitation of 440 nm with emission at 482 nm (22).

**Congo red spectroscopic amyloid assay.** Congo red (Sigma; 0.7 mg/ml in 5 mM potassium phosphate, 150 mM NaCl, pH 7.4) was filtered through a 0.2-μm syringe filter prior to use, and the spectrum was obtained between 400 nm and 800 nm at 4°C (21).
Values greater than 5% are considered to be predictive of amyloid formation (6a). The rest of the protein did not show high propensity for β-aggregation. Two other Als adhesins, Als1p and Als3p, have identical sequences and similar predicted β-aggregation potentials in this region (Table 1).

We obtained a synthetic 13-mer peptide with the sequence of Als5p residues 322 to 334, including the amyloid-predicted residues 325 to 331. On suspension and stirring, this peptide formed a gel, a positive sign of the presence of amyloid fibrils (14, 22). The gelled peptide bound Congo red, enhancing the absorbance about twofold (characteristic of amyloids) and also redshifting the absorbance peak to 541 nm (Fig. 3A) (6). A similar sample of the peptide that had not been stirred did not significantly affect the Congo red spectrum. The gelled peptide also enhanced the fluorescence of thioflavin T, enhancing fluorescence twofold and shifting the emission peak from 481 nm to 478 nm (Fig. 3B). Here also, such enhancement and shifting are characteristic of amyloids (6, 22).

The aggregated peptide was negatively stained with uranyl acetate and inspected by electron microscopy. After sonication, the aggregated peptide formed a meshwork of unbranched straight linear fibrils with diameters of ∼22 to 30 nm (Fig. 4A). In some cases, there were helical markings within the fibers, similar to those seen in many amyloids (Fig. 4A3) (11). The fibers were also visualized by atomic force microscopy (data not shown).

We also obtained a similar peptide with a V326N substitution. This mutated peptide had only a 4% β-aggregation potential according to TANGO, but it had similar β-strand propensity (18). The peptide did not form a gel, nor was it fibrillar, nor did it alter the spectrum of Congo red (Fig. 3B) or thioflavin T (data not shown).

Als5p forms amyloids. Amyloid formation of the peptide implied similar properties in the Als5p protein itself. Suspensions of two purified soluble forms of the protein, Als5p20-431 and Als5p20-664, aggregated to form a visible precipitate upon concentration to 0.5 mg/ml or above. To confirm that the aggregates were composed of Als5p, aggregates from Als5p20-431 and Als5p20-664 were washed and suspended in phosphate buffer, sonicated, and run in parallel on 4 to 20% native polyacrylamide gels. The low-mobility aggregates were stained with Coomassie blue and contained the V5 epitope that had been added in their construction (Fig. 2A) (24). These aggregates were partially dissociated in the presence of SDS, yielding a ladder of multimeric species with apparent molecular masses.

### RESULTS

Amyloid formation by a peptide from Als proteins. Purified soluble forms of Als5p spontaneously aggregated at concentrations greater than 0.5 mg/ml. These aggregates were not solubilized by sodium dodecyl sulfate (SDS) (Fig. 2) or guanidine HCl (data not shown). We therefore tested Als5p for amyloid-forming sequences with TANGO, a program that predicts the tendencies of polypeptide sequences to form β-strand-rich aggregates based on inter- and intramolecular interaction energies (6a). Regions predicted to form such aggregates show a high correlation with amyloid-forming regions in many proteins (18). TANGO predicted that a heptapeptide sequence, I325VIVATT331, within the T region of Als5p would partition into a β-aggregate with a value of 93% (Fig. 1).

![Image](https://example.com/image.png)

**FIG. 2.** Gel electrophoresis of Als5p. All gels were 4 to 20% acrylamide gradient gels. (A) SDS gel of partially solubilized precipitates from preparations of purified Als5p fragments stained with Coomassie blue (left) or immunoblotted with anti-V5 (right). The precipitates were isolated and washed three times before electrophoresis. The illustrated region of the gel was immediately below the loading wells. (B) Immunoblot of Als5p20-664 showing multimeric aggregates in the presence of SDS. The protein was concentrated 10-fold after purification, and an aliquot of the suspension was treated with SDS and then electrophoresed for 24 h. The filled arrowheads mark the positions of Bio-Rad Rainbow molecular weight markers. The open arrowhead marks the Als5p20-664 monomer with an apparent molecular mass of 120 kDa (24).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence position</th>
<th>Sequence</th>
<th>β-Aggregation potential (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS5</td>
<td>325</td>
<td>IVIVATT</td>
<td>93</td>
</tr>
<tr>
<td>ALS3</td>
<td>325</td>
<td>IVIVATT</td>
<td>90</td>
</tr>
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<td>325</td>
<td>IVIVATT</td>
<td>90</td>
</tr>
<tr>
<td>ALS9</td>
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<td>IVVTT</td>
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<td>ALS2</td>
<td>324</td>
<td>FVIVATT</td>
<td>93</td>
</tr>
<tr>
<td>ALS7</td>
<td>358</td>
<td>TILVL</td>
<td>80</td>
</tr>
</tbody>
</table>

a Amino acid sequence position of the first residue.
b TANGO β-aggregation potential.
FIG. 3. Absorbance and fluorescence spectra. (A) Congo red absorbance spectrum (solid line) and spectrum of Congo red in the presence of the unstirred “unaggregated” peptide S23-NGIVIVATTTRTV33 (dotted line) or the aggregated peptide S23-NGIVIVATTTRTV33 (dashed line). The peptide concentration was 20 μg/ml. (B) Congo red absorbance spectra. The curve with maximal absorbance is in the absence of added peptide. Adding the “mutant” peptide SNGINIVATTTRTV that had been incubated with or without stirring slightly diluted the dye but did not otherwise change the spectrum (superimposed spectra with slightly lower absorbance maxima). The peptide concentration was 20 μg/ml. (C) Fluorescence emission spectra for thioflavin T (solid line), thioflavin T in the presence of an unstirred “unaggregated” peptide (dotted line), and thioflavin T in the presence of aggregated peptide (dashed line). The peptide concentration was 6.6 μg/ml. (D) Congo red difference spectra for unaggregated (solid line) or aggregated (dashed line) Als5p20-431. Absorbance values for Congo red alone have been subtracted (maximal absorbance, 0.0510 at 489 nm). The final concentration of protein was 5 μg/ml. (E) Congo red difference spectra for unaggregated (solid line) or aggregated (dotted line) Als5p30-664. Absorbance values for Congo red alone have been subtracted (maximal absorbance, 0.0646 at 502 nm). The final concentration of protein was 3.5 μg/ml.
ranging from 120 kDa (the monomer size) to over 1 million Da (Fig. 2B). These protein aggregates also enhanced the absorbance of Congo red, a characteristic used to define amyloids (6, 22). Difference spectra illustrate this effect (Fig. 3D and E) and show that both secreted proteins increased the absorbance, with suspensions containing aggregates having a greater effect than suspensions without detectable aggregates. Aggregated Als5p20-431 enhanced the peak absorbance by 38%, as expected for an amyloid. Aggregates of the larger fragment, Als5p20-664, increased absorbance 61% and also redshifted the peak absorbance from 501 nm to 520 nm (Fig. 3D). Such redshifting is also characteristic of amyloids (6, 22).

Als5p20-431 and Als5p20-664 aggregates were stained with 2% uranyl acetate and inspected by electron microscopy. The aggregates were clumps of small, rounded structures with diameters of 50 to 100 nm (Fig. 4B1) that yielded fibers on sonication (all other structures in Fig. 4B and C). Als5p20-431 showed two types of fibrillar morphologies with widths from 30 nm to 90 nm (Fig. 4B2 to B4). Some fibrils were branched, and some showed uneven thickness along the length of the fibril and apparent globular regions. Aggregated Als5p20-664 was present in bundles of diverse fibrils with diameters between 50 nm and 240 nm (Fig. 4C1 to C4). There were at least three morphologies, including one (Fig. 4C3) that looked like a linear aggregate of the 50- to 100-nm particles shown in Fig. 4B1.

**Evolutionary conservation of amyloid-forming sequences.** Conservation or divergence of sequences can be estimated as $K_a/K_s$, the ratio of nonsynonymous to synonymous nucleotide changes (21). We determined this ratio for each of the regions of Als, using comparisons between the three close paralogs ALS1, ALS3, and ALS5. The Ig-like regions showed moderate conservation, with average $K_a/K_s$ values of 0.28 to 0.3 (Table 2), slightly above the genomic averages for Saccharomyces orthologs (26) and for human-chimpanzee orthologs (4). The higher ratio is expected for paralogous sequences like ALS, because they usually diverge faster than orthologs (21). In contrast, the T regions showed very few nonsynonymous substitutions and had $K_a/K_s$ values 5.2-fold lower. TR regions were also conserved, with a ratio 3-fold lower than the Ig-like regions. The stalk regions had high $K_a/K_s$ ratios characteristic of evolution on sequences with few selective constraints. Thus, the T regions are extremely highly conserved and are under apparent selection to retain specific sequences, including those with amyloid-forming ability. That is, the T regions vary less than any other parts of the Als proteins. There are similar amyloid-forming sequences in homologous positions in other Als paralogs (Table 1).

**DISCUSSION**

Als5p amyloids. The Als adhesins cause adherence to host ligands and fungal aggregation. The aggregation reaction has properties of amyloid-like interactions (25). Our observations imply that these amyloid-like properties are a manifestation of underlying amyloid-like protein-protein interactions. Three fragments of Als5p formed amyloids under native-like conditions. A tripeptide from the T region bound Congo red and thioflavin T and also formed helical fibers with 22- to 30-nm diameters. Two large adherence-active truncations of Als5p, with 412 and 625 residues after removal of the signal sequence, also had dye-binding ability and aggregated as fibers with amyloid-like morphologies. Thus, Als5p fragments formed amyloid structures, and they formed at room temperature or at 4°C, at neutral pH, and at submilligram concentrations.

**Role of amyloid in adherence.** The structures of Als proteins are compatible with known properties of authentic amyloids. The best-characterized amyloid structures consist of parallel $\beta$-sheets composed of identical sequences from many protein molecules (11, 27, 32). The individual strands run perpendicular to the fiber axis, with the $\beta$-sheets themselves parallel to the fiber axis. These characteristics are correlated with dye binding and spectral shifting for Congo red and thioflavin T (6, 22).
local interaction of Als adhesins, both between adhesins and between adhesins and the wall matrix. The stalks are 300 residues long, and the longer stalk in Als5p would exceed 200 nm (12, 13). Thus, there is considerable freedom to allow intermolecular interactions.

The 300-residue stalks in Als1p and Als3p would have lengths of 60 nm or more, and the longer stalk in Als5p would exceed 100 nm (12, 13). Thus, there is considerable freedom to allow local interaction of Als adhesins, both between adhesins anchoring in a single cell (cis) and between apposed cells (trans interactions). On the other hand, the tethered nature of the adhesin limits amyloid interaction to a local region, so it is likely that in vivo amyloid molecular aggregates would be too small to visualize by TEM.

### Conservation of the amyloid-forming region.

The T region is the most strongly conserved part of the Als proteins and shows over 90% identity within the gene family, whereas other regions are as low as 30 to 40% (12, 13). The $K_r$ values for the T region are similar to those for the Ig region, a similarity that shows a similar divergence times for the two regions. In contrast, the very low $K_r$ values in the T region show that many fewer mutations are tolerated in the T region than in the Ig-like region. In other words, the T-region sequences are under intense purifying selection. (Note that our evolutionary analysis was done using paralogs from the same genome, which tend to diverge faster than the orthologs we have used for comparisons [26], so the conservation is all the more unusual.) Conserved T-region sequences are therefore present in each Als protein as it is differentially expressed in growth and infection (10, 28, 33, 34).

The high $K_r$ values in the TR and stalk regions indicate higher rates of recombination in these regions, as noted in other analyses (13, 35, 36). These less strongly selected regions have accumulated many mutations since the divergence of the Als genes (13, 36).

Amyloid states of proteins have long been implicated in degenerative diseases, but few biological processes are known to use amyloids in normal cellular functions. We have documented amyloid-like interactions in Als adhesins from *C. albicans* and the evolutionary conservation of the sequences that confer this ability. Therefore, amyloid formation could be a part of the normal adherence function of Als proteins, which also adhere through other, more traditional types of interaction, as well. Such amyloid-forming abilities may be part of the armamentarium of adherence strategies for eukaryotic microorganisms, as well as for gram-negative bacteria.

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### Table 2. Ka/Ks ratios for specific regions of *ALS1*, *ALS3*, and *ALS5*

<table>
<thead>
<tr>
<th>Region of <em>ALS</em></th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>$K_a$</th>
<th>$K_s$</th>
<th>$K_a/K_s$</th>
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<tr>
<td>Ig-like</td>
<td><em>ALS5</em></td>
<td><em>ALS1</em></td>
<td>0.1084</td>
<td>0.3499</td>
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<td></td>
<td><em>ALS5</em></td>
<td><em>ALS3</em></td>
<td>0.1295</td>
<td>0.4210</td>
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<tr>
<td></td>
<td><em>ALS1</em></td>
<td><em>ALS3</em></td>
<td>0.1064</td>
<td>0.4643</td>
<td>0.2291</td>
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</tbody>
</table>

Anchored in a single cell (cis) and between apposed cells (trans interactions). On the other hand, the tethered nature of the adhesin limits amyloid interaction to a local region, so it is likely that in vivo amyloid molecular aggregates would be too small to visualize by TEM.

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22). Because the Als5p peptide S$^{32}$NGIVIVATRTV$^{334}$ gelled and formed fibers with typical amyloid morphology and dye binding, it should conform to this structural model. The fibrillar morphology and dye-binding ability of the larger Als5p proteins imply that these characteristics are like those present in the amyloids formed by other multidomain proteins, such as RNase (27).

Als-mediated adherence also matches amyloid formation in its Als-to-Als homologous-binding characteristics. As in amyloid interactions, immobilized Als5p is a ligand for other Als5p molecules, as shown in an enzyme-linked immunosorbent assay-like assay of surface-displayed Als5p (24). Because the Als5p self-binding reaction is not saturable (24, 25), these interactions are like amyloid “seeding,” the process in which one or a few molecules in the amyloid conformation nucleate the addition of supramolecular insoluble aggregates (Fig. 2 and 4) (18, 19). The interaction is broader than Als5p, because Als5-displaying cells aggregate with yeast cells expressing other Als proteins, as well (15). Because Als1p, Als3p, and Als5p share the same amyloid-forming sequence, they may be able to form mixed amyloids in vivo, a state that would be consistent with the broad specificity of Als-Als binding and the amyloid-like state of adherence. Als2p may also be able to coaggregate, because its sequence varies at only one position at the end of the amyloid-forming region (Table 1). Therefore amyloid formation is likely to be present in other Als proteins (Table 1).

### Conditions for amyloid formation.

Als5p amyloids formed under native-like conditions at low concentrations (500 μg/ml), so similar structures are likely to form during adherence. In fact, cell surface concentrations are much higher than the solution concentrations used to form amyloids in vitro. Surface adhesin concentrations may be as much as 20 mg/ml within a 200-nm spherical shell at the outer surface of the wall (5). The Als adhesins, though tethered covalently to the wall matrix, have long, flexible stalks that allow intermolecular interactions. The 300-residue stalks in Als1p and Als3p would have lengths of 60 nm or more, and the longer stalk in Als5p would exceed 100 nm (12, 13). Thus, there is considerable freedom to allow local interaction of Als adhesins, both between adhesins anchored in a single cell (cis) and between apposed cells (trans interactions). On the other hand, the tethered nature of the adhesin limits amyloid interaction to a local region, so it is likely that in vivo amyloid molecular aggregates would be too small to visualize by TEM.

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