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Heterogeneous Assembly of Complementary Peptide Pairs into Amyloid Fibrils with $\alpha - \beta$ Structural Transition

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KEYWORDS:

aggregation · amyloid fibrils · electrostatic interactions · peptides · protein structures

Intermolecular self-assembly of a large number of polypeptide chains into macromolecular constructs occurs widely in biological systems. One of such macromolecular self-assemblages of great interest is the amyloid fibril.[1] The amyloid fibril is a misfolded and undesirable state for proteins as biomolecules, since it has been proposed to be a causative agent for a variety of fatal diseases known as amyloid diseases, such as Alzheimer's disease and prion diseases.[2] However, it is considered that the fibril has a highly ordered quaternary structure, in which numerous β-stranded polypeptide chains align regularly,[1] and thus this kind of fibril has the potential to be engineered into proteinaceous materials.[3–6] Amyloid fibril primarily comprises a single polypeptide species, that is, it is a homogeneous self-assemblage. Here we report the heterogeneous assembly of designed peptides into amyloid fibrils accompanied by a drastic secondary structural transition from an α helix to a β sheet. The heterogeneous assembly into fibrils is accomplished by complementary electrostatic interactions between pairs of peptide species, each of which is not able to self-assemble.

The design of peptides that could heterogeneously assemble into amyloid fibrils commenced by engineering our de novo

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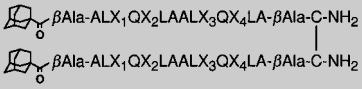
designed peptides that homogeneously self-assembled into fibrils.[7] The peptides undergo a self-initiated structural transition from an α helix to a β sheet in neutral aqueous solution, and simultaneously self-assemble into fibrils in an autocatalytic manner.[7] A coiled-coil structure composed of two amphiphilic α helices[8] with double-heptad repeats $(ALEQKLA)_2$ was designed (this parent sequence was named 7.EKEK, Table 1). The two polypeptide chains were linked by a disulfide bond between cysteine residues at the C termini to maintain a parallel orientation of the two α helices. Although an ideal amphiphilic α helix was designed, the sequence also had the potential to form an amphiphilic β strand.[8] A 1-adamantanecarbonyl (Ad) group was attached to the N terminus of the peptide and thus exposed to the solvent, thus inducing intermolecular peptide association through hydrophobic interactions.[7a] The amino acid sequence of the original peptide 7.EKEK[7a] contains four charged residues; two glutamic acid (negatively charged) and two lysine residues (positively charged) (Table 1). In the present study, we manipulated these charged residues as the complementarity-determining residues for the assembly of the peptide into fibrils. To this end, we prepared all types of Ad-linked peptides bearing glutamic acid or lysine residues at positions X_1 to X_4 (Table 1). Conformational analysis of these peptides was carried out by circular dichroism (CD) spectroscopic, transmission electron microscopic (TEM), and amyloid-specific dye binding studies. The CD method provides information concerning secondary structure, and TEM enables a direct observation of peptide aggregates. A quantitative analysis of the amyloid fibril formation was carried out by using an amyloid-specific dye, thioflavin T (ThT), which associates with amyloid fibrils, and the binding results in a significant increase in the fluorescence depending on the amount of fibrils present.[9]

Table 1. *Primary structures of the designed peptides.*



Peptide	X_1	X_2	X_3	X_4	Total charge at neutral pH
1.EEEE	E	E	E	E	−8
2.EEEK	E	E	E	K	−4
3.EEKE	E	E	K	E	−4
4.EKEE	E	K	E	E	−4
5.KEEE	K	E	E	E	−4
6.EEKK	E	E	K	K	0
7.EKEK	E	K	E	K	0
8.EKKE	E	K	K	E	0
9.KEEK	K	E	E	K	0
10.KEKE	K	E	K	E	0
11.KKEE	K	K	E	E	0
12.EKKK	E	K	K	K	+4
13.KEKK	K	E	K	K	+4
14.KKEK	K	K	E	K	+4
15.KKKE	K	K	K	E	+4
16.KKKK	K	K	K	K	+8

First, a conformational analysis of each peptide was carried out. CD studies revealed that all peptides predominantly formed an α helix or a random-coil structure (depending on their sequences) shortly after dissolution in the neutral buffer (at 25 °C, peptide concentration 12 μM). No β-sheet structure was observed in the initial stage. Since intermolecular association of the peptides is required for the formation of β-sheet fibrils, it is expected that neutral peptides assemble into fibrils more readily than negatively or positively charged peptides. Indeed, examples in which a time-dependent transformation to β-sheet fibrils was observed were limited to neutral peptides, but charge neutralization was not sufficient for β-sheet fibril formation. The CD, TEM, and ThT-binding studies revealed that among the six neutral peptides (6.EEKK to 11.KKEE), four peptides, 6.EEKK, 7.EKEK, 10.KEKE, and 11.KKEE, were able to self-assemble into β-sheet fibrils.[10] However, the time required for β-sheet formation and the amount of fibrils varied depending on the peptide sequence (Figure 1; see Supporting Information for details of the CD data). The other two neutral peptides, 8.EKKE and 9.KEEK, were not able to form β-sheet fibrils even after four days (Figure 1). These results suggest that the positions of positive and negative charges are critical for the well organized assembly of β strands, and that these four peptides have self-complementary sequences which enable them to homogeneously self-assemble into fibrils (discussed below). None of the negatively (1.EEEE to 5.KEEE) or positively charged peptides (12.EKKK to 16.KKKK) was able to form β-sheet fibrils (Figure 1 and Supporting Information),[11] which is likely due to their unfavorable intermolecular interactions that prevent association.

Next, from all possible pairs of the 16 peptides (120 combinations), we searched complementary pairings that enabled heterogeneous assembly of two peptide species into

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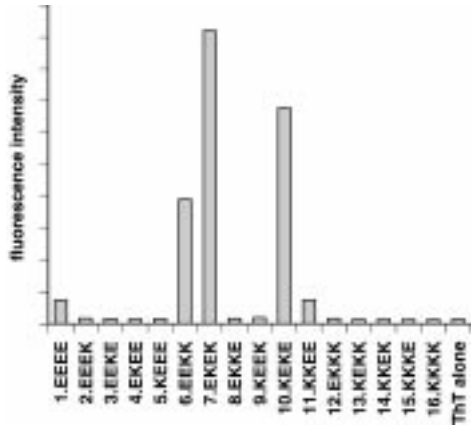

Figure 1. *Intrinsic ability of the peptides to self-assemble into fibrils. Fibril formation of each peptide alone (after 4 d incubation at 12 μM peptide concentration in 20 mM Tris-HCl buffer (pH 7.4)/2.5 % TFE at 25 °C) was examined by ThT-binding analysis. The fluorescence intensities (in arbitrary units) of ThT at 480 nm (λ_{ex} = 440 nm) in the presence or absence of the peptide are shown.*

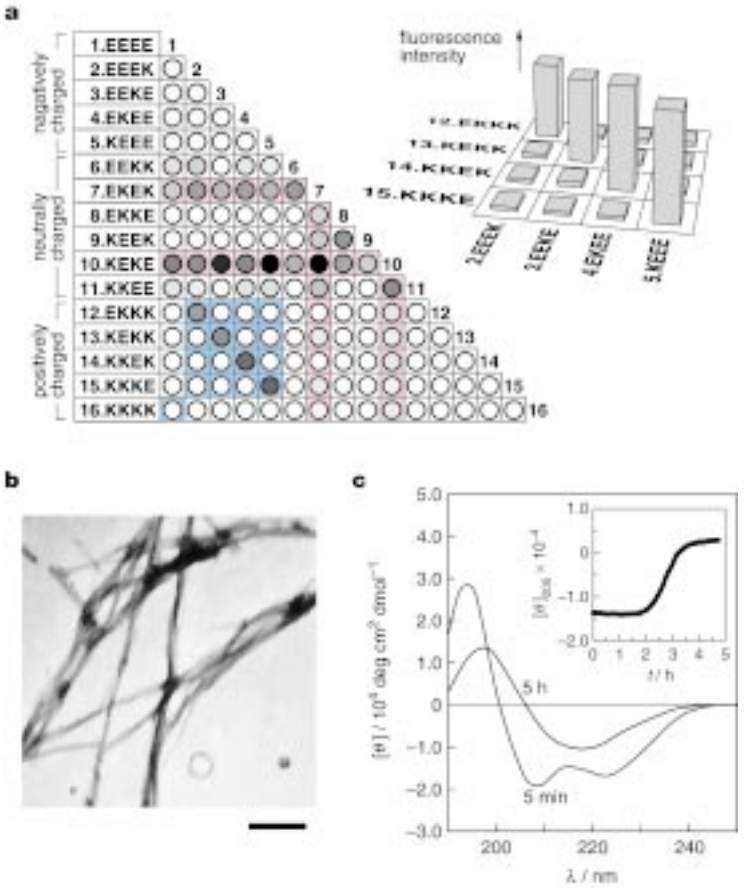

Figure 2. *Fibril formation of mixtures of two peptide species. a) Screening of pairings of two complementary peptide species that are capable of assembling heterogeneously into fibrils. Fibril formation of the two-peptide mixtures (after 24 h incubation in the neutral buffer at 25 °C, 6 μM concentration of each peptide) was examined by ThT-binding analysis. The fluorescence intensities of ThT in the presence of the mixtures are represented by the black color density of the circles, and a region of interest is redrawn on the right hand side. The combinations of negatively and positively charged peptides that result in a neutral net charge are colored in blue, and the combinations containing 7.EKEK or 10.KEKE, which have a higher potential to form the fibrils individually, are colored in magenta. b) Transmission electron micrograph of β-sheet fibrils formed by the 4.EKEE/14.KKEK mixture, which was incubated in the buffer for 24 h and then negatively stained. Scale bar length is 200 nm. Similar images were obtained for each of the pairs 2.EEEK/12.EKKK, 3.EEKE/13.KEKK, and 5.KEEE/15.KKKE. c) Time-dependent CD spectral changes of the 5.KEEE/15.KKKE mixture. The time course of the transition monitored by [θ] at 205 nm is shown in the inset. Similar CD data were obtained for each of the pairs 2.EEEK/12.EKKK, 3.EEKE/13.KEKK, and 4.EKEE/14.KKEK.*

amyloid fibrils. Two species were mixed at equimolar ratios (6 μM each) and incubated in neutral buffer for 24 h, and then fibril formation was examined by ThT-binding analysis (Figure 2a). The combinations of interest are those of negatively and positively charged peptides that neutralize the net charge (blue region in Figure 2a). Since none of them is able to form β-sheet fibrils individually (see Figure 1), assembly into fibrils is expected only when another peptide species is present in solution. From the combinations making up the blue region in Figure 2a, four specific pairings were selected as fibril-forming ones. The TEM study confirmed that these mixtures indeed formed fibrillar assemblages (Figure 2b), which had a morphology similar to that of the amyloid fibrils formed by naturally occurring proteins such as β-amyloid or prion proteins.[12] The CD studies revealed that each of the selected four pairs formed an α-helix structure initially, and changed to a β-sheet structure spontaneously. The α-to-β structural transitions of these pairs, 2.EEEK/12.EKKK, 3.EEKE/13.KEKK, 4.EKEE/14.KKEK, and 5.KEEE/15.KKKE, were completed within 2, 8, 8, and 5 h, respectively (Figure 2c), whereas each species alone existed predominantly as an α-helix or random-coil structure over 4 d. For each of the four pairs, the α-helicity of the mixture was higher than that of solutions containing the single species, suggesting that the two species interact with each other prior to β-sheet fibril formation (difference in $[\theta]_{222}$ between the spectrum of the mixture and the sum spectrum of each species: 2.EEEK/12.EKKK, 5200; 3.EEKE/13.KEKK, 5400; 4.EKEE/14.KKEK, 4400; 5.KEEE/15.KKKE, 12 000 deg cm^2 dmol^{-1}). The α-to-β structural transition profiles of these mixtures as monitored by the molar ellipticity at 205 nm are quite sigmoidal (Figure 2c, inset). This suggests that the transitions are autocatalytic as proposed in the α-to-β structural transition and subsequent fibril formation of prion proteins.[2a, 7] Additional fibril-forming pairing was observed for the combination 8.EKKE/9.KEEK (Figure 2a), in which each species was neutral but unable to form fibrils individually (Figure 1). The CD study revealed that the 8.EKKE/9.KEEK mixture underwent the α-to-β structural transition within 8 h, and TEM provided similar images as for the aforementioned four pairings.

The results shown in Figure 2a clearly demonstrate that there are complementary pairings of the peptides which enable them to assemble heterogeneously into amyloid fibrils. From the fibril-forming combinations 2.EEEK/12.EKKK, 3.EEKE/13.KEKK, 4.EKEE/14.KKEK, 5.KEEE/15.KKKE, and 8.EKKE/9.KEEK we observed a trend explaining why these pairings are compatible. As shown in Figure 3a, in all five cases, if the two species are aligned inversely, they are able to form negative – positive charge pairings. Thus,

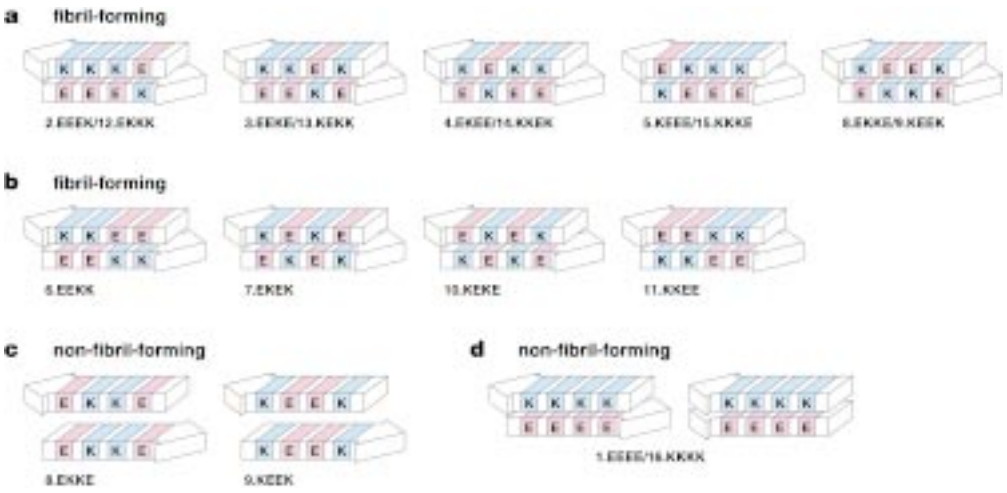

Figure 3. *Schematic representation of heterogeneous or homogeneous assembly of complementary peptides. The complementarity required for the assembly is determined by simple negative – positive ion pairing, by which the well-defined molecular orientation is accomplished. a) Heterogeneous fibril formation by pairing of two complementary peptides. b) Homogeneous fibril formation by pairing of self-complementary peptides. c) No fibril formation is observed for neutral peptides because of their palindromic sequences. d) The pairing of positively and negatively charged peptides results in a neutral net charge but does not lead to fibril formation because the unique molecular orientation is not defined.*

two species of β strands would be arrayed antiparallelly to form ion pairs in the fibrils. This trend also illustrates why some neutral peptides are able to form homogeneous fibrils, but others are not (Figure 1). The homogeneous fibril-forming peptides, 6.EEKK, 7.EKEK, 10.KEKE, and 11.KKEE, can form ion pairs intermolecularly if the strands are aligned antiparallelly, meaning that their sequences are self-complementary (Figure 3 b). On the contrary, in the cases of the neutral but non-fibril-forming peptides 8.EKKE and 9.KEEK, charge repulsion could occur in both parallel and antiparallel arrangements due to their palindromic sequences, so that the assembly of β strands would be disfavored (Figure 3 c). In addition, we speculated why the pairing of 1.EEEE/16.KKKK failed to form fibrils (Figure 2 a). Ion pairing of these two species is possible in both parallel and antiparallel arrangements, and thus the unique orientation which is apparently critical for the assembly into fibrils cannot be defined (Figure 3 d). However, from this explanation, the pairing of 8.EKKE/9.KEEK should fail to form fibrils, yet it enables fibril formation. The critical difference between the pairs 1.EEEE/16.KKKK and 8.EKKE/9.KEEK is the secondary structure in the initial stages. 1.EEEE/16.KKKK is present predominantly as a random coil, whereas 8.EKKE/9.KEEK initially forms an α helix. The α-helix formation in the initial stage might be effective for inducing intermolecular associations that result in the required molecular orientation.[7] An intermolecularly antiparallel arrangement between two peptide species in the fibrils is suggested, while the intramolecular arrangement of the two strands is not clear. Although it has been suggested that the polypeptide chains in amyloid fibrils adopt a cross-β structure in which the β strands are aligned perpendicularly to the fiber axis, the precise molecular details of the structure have not been fully resolved yet.[1, 13, 14] Both antiparallel and parallel β-sheet arrangements in fibrils have been proposed.[14]

From a biological standpoint, compounds that inhibit fibril formation are interesting, since they might be research targets for the development of therapies against amyloid diseases.[15] Examining all possible combinations of peptide pairs, it was found that fibril formation by 7.EKEK and 10.KEKE, which had a higher potential to self-assemble into fibrils, was inhibited by the positively charged peptides (12.EKKK to 16.KKKK), but not by the negatively charged ones (magenta region in Figure 2 a). Although it is not clear why fibril formation was inhibited selectively by the positively charged peptides, this result may prove to be useful for the design of amyloid formation inhibitors.

We have achieved the heterogeneous assembly of two peptide species into β-sheet fibrils. The complementary heterogeneous assembly is accomplished by simple ion pairings. The results presented here may provide a new method for constructing a heterogeneously assembled polypeptide fibril composed of multiple species, in which the alignment and orientation of each species are highly ordered. The introduction of different multifunctional groups into this construct may be possible, leading to nanoscale materials with novel functional, physicochemical, and mechanical properties.[3–6] Furthermore, the peptides assemble into fibrils while undergoing drastic secondary structural transition from α helix to β sheet, which mimics one of the most critical characteristics of prion proteins.[2c] To efficiently study the fine details of protein organization, the use of simplified model peptides like those presented here leads to a clearer understanding of underlying mechanisms whereby conformational changes and the aggregation/assembly of proteins occur. Such information may divulge a system for clarifying and controlling off-pathway aggregation of naturally occurring proteins.

Experimental Section

Peptide synthesis: Peptides were synthesized by the solid-phase method using standard fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry,[7] and the intermolecular disulfide bond was formed in DMSO/trifluoroacetic acid (1:9, v/v) solution.[16] The peptides were purified by reversed-phase HPLC and identified by MALDI-TOF mass spectrometry and amino acid analysis.

CD spectroscopy: Each peptide was dissolved in trifluoroethanol (TFE) at 0.48 mM. The measurements were started immediately after dilution of the TFE solution with 20 mM Tris-HCl buffer (pH 7.4). Final concentrations of the peptides were 12 μM and the TFE content was 2.5 %. The concentration of the peptide solutions were determined by quantitative amino acid analysis. We have confirmed that TFE as

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the stock solvent does not significantly affect the conformational properties of the peptides. For example, when the lyophilized powder of 7.EKEK was directly dissolved in the buffer, that is, without TFE, the peptide initially formed an α-helical structure which gradually changed to the β-sheet fibrils, as was the case when using TFE as the stock solvent. Thus, the initial α-helix structure is not induced by TFE. For quantitative sample preparations, TFE stock solutions were used. CD spectra were measured by using a quartz cell with 1.0 mm path length, and recorded in terms of mean residual molar ellipticity ($[\theta]$ deg cm^2 dmol^{-1}).

Thioflavin T(ThT)-binding analysis: Peptide solutions were prepared and incubated as described for the CD measurements. After the indicated incubation periods, the ThT solution (240 μM in water) was added to the peptide solution, after which fluorescence measurements were carried out.[7, 9] For the measurements shown in Figure 1, fluorescence emission spectra of solutions of 6 μM ThT in the presence of 12 μM peptide were recorded at an excitation wavelength of 440 nm by using a 5 × 5 mm quartz cell. For the measurements shown in Figure 2a, fluorescence intensities of solutions of 20 μM ThT in the presence of 11 μM peptide (final volume 330 μL) were recorded on a multi-well plate reader (excitation filter, 425 – 475 nm; emission filter, 525 – 535 nm) using a 96-well plate.

TEM study: Peptide solutions were prepared and incubated as described for the CD measurements. The sample was adsorbed to a carbon-coated copper grid and then negatively stained with a 2% (w/v) aqueous phosphotungstic acid solution.

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[11] Although ThT in the presence of 1.EEEE showed a little fluorescence enhancement, similar in degree to that observed for 11.KKEE (Figure 1), CD and TEM studies revealed that 1.EEEE was predominantly present in the random-coil conformation during the observation period and that there was no fibrous aggregate. Thus, we concluded that 1.EEEE was not capable of forming β-sheet fibrils. In contrast, 11.KKEE was shown to form fibrils by TEM analysis, although its CD spectrum after 4 d incubation was not typical for a β-sheet structure and the fluorescence intensity of ThT was not very high.

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Selective Cleavage of Unpaired Uridines with a Tyrosine – Cyclen Conjugate

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Small molecules that selectively cleave RNA could not only act as important tools in molecular biology, they also have the potential for interfering with the life cycle of cells. These compounds could be developed into therapeutic agents. In this context we described the selective cleavage of the transactivation response element of HIV-1 RNA (TAR RNA) by the conjugate **1** in which the arginine-rich region of the transactivator protein Tat is covalently attached to cyclen (1,4,7,10-tetraazacyclododecane).[1] The only cleavage site was located between bases U 31 and G 32, as can be seen by comparing the results of the gel electrophoresis after alkaline hydrolysis and RNase T1 digestion with those of the cleavage by **1** (Figure 1a; lanes 6, 4, and 1, respectively). In the course of mechanistic investigations of the

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