Amyloid Architecture: Complementary Assembly of Heterogeneous Combinations of Three or Four Peptides into Amyloid Fibrils

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The amyloid fibril is a misfolded and undesirable state for proteins that has been proposed to be a causative agent for a variety of fatal diseases known as amyloid diseases, such as Alzheimer's and prion diseases. However, the fibril has a highly ordered tertiary structure in which numerous β-strand polypeptide chains align in a regular pattern. Thus, this kind of fibril has the potential to be engineered into proteinaceous materials. Amyloid fibrils of misfolded proteins primarily comprise a single polypeptide species, that is, the self-assembly is homogeneous. We here found that three or four designed peptides can assemble heterogeneously and cooperatively into amyloid fibrils, a process accompanied by a drastic secondary structural transition from α helix to β sheet. Heterogeneous assembly into fibrils is accomplished by complementary electrostatic interactions between three or four peptide species, each of which is not able to self-assemble homogeneously. These findings will lead to a novel way to study the molecular details of amyloid formation and also to design β-sheet peptidyl materials.

KEYWORDS:

amyloid fibrils · peptides · protein structures · self-assembly

Introduction

Amyloid fibril formation by misfolded proteins has attracted the attention of both the medical and engineering communities and is of relevance to a variety of fatal diseases, which include Alzheimer's and prion diseases.[1, 2]. An improved understanding of protein misfolding as well as clarification of the folding pathways is critical to the study of proteins involved in these diseases. The pathway of protein misfolding involves a conformational transition, such as that from α helix to β sheet. This transition is especially apparent in the transformation of the α-helix-rich cellular form of the prion protein (PrP^C) to the scrapie isoform with higher β sheet content (PrP^{Sc}).[1, 3] The amyloid fibril has a highly ordered tertiary structure in which numerous β-strand polypeptide chains align regularly[1, 2, 4] and there are therefore promising prospects for the application of this macromolecular construct in the use of proteinaceous materials.[5–9] Amyloid fibrils of misfolded natural proteins primarily comprise a single polypeptide species, that is, the self-assembly is homogeneous. It is therefore of great value to the development of novel biomaterials to establish a method for the fabrication of amyloid fibrils from multiple peptide species.[10e]

We recently reported the design of peptides that undergo a self-initiated structural transition from an α helix to a β sheet in a neutral aqueous solution and self-assemble into amyloid fibrils (Figure 1).[10] A series of these peptides is composed of two amphiphilic α helices with double-heptad repeats $(ALEQKLA)_2$. The two peptide chains are linked by a disulfide bond between Cys residues at the C-termini. Although an ideal amphiphilic α helix[11] has been designed, the sequence created also has the potential to form an amphiphilic β strand[11] in which hydrophobic residues and charged residues are separated on different faces of each heptad. A 1-adamantanecarbonyl (Ad) group was attached to the N terminus of the peptide and exposed to the solvent, which caused intermolecular peptide associations through hydrophobic interactions.[10a] In order to accomplish complementary assembly by multiple peptide species, the charged residues were manipulated as if they were complementarity-determining residues. A charge-distributed peptide library composed of Ad-linked peptides that bear E(−) or K(+) residues at positions X_1–X_4 ($2^4 = 16$ compounds) was thus prepared (Figure 1).[10e] Previously, we demonstrated that two peptide species in this library undergo complementary assembly into fibrils, although each is unable to assemble into fibrils individually.[10d,e] In the present study, we attempted to expand the amyloid architecture to one that uses three or four complementary peptide species laminated into amyloid fibrils. We present, for the first time, a novel approach for the construction of amyloid fibrils that involves complementary assembly of three or four peptide species. Combined with the assembly of the two different peptides in our previous study,[10d,e] various applications that use two, three, and four different β strands can be designed for the development of fibrous peptide

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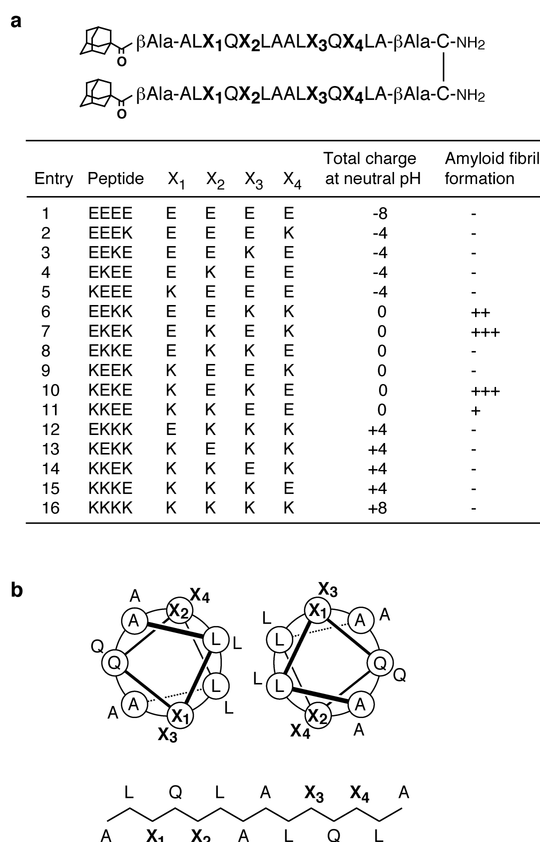

Entry	Peptide	X_1	X_2	X_3	X_4	Total charge at neutral pH	Amyloid fibril formation
1	EEEE	E	E	E	E	-8	-
2	EEEK	E	E	E	K	-4	-
3	EEKE	E	E	K	E	-4	-
4	EKEE	E	K	E	E	-4	-
5	KEEE	K	E	E	E	-4	-
6	EEKK	E	E	K	K	0	++
7	EKEK	E	K	E	K	0	+++
8	EKKE	E	K	K	E	0	-
9	KEEK	K	E	E	K	0	-
10	KEKE	K	E	K	E	0	+++
11	KKEE	K	K	E	E	0	+
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	-

Figure 1. a) Designed peptides in the peptide library and their ability to form amyloid fibrils individually, examined by ThT binding and TEM studies[10e]. b) A helix wheel drawing as a coiled-coil form and a β-strand drawing of the core 14-residue peptide.

materials with a defined two- or three-dimensional structure. This method makes possible the sequential array of multiple kinds of functional groups and their site-specific incorporation into the predeterminable fibrous scaffold. Moreover, this kind of study provides a new insight into the molecular details possibly involved in amyloid formation by disease-related proteins.

Results and Discussion

Amyloid fibrils from three peptide species

First, the complementary triplets from the peptide library that heterogeneously assemble into fibrils were identified. The charge-distributed peptide library composed of Ad-linked peptides that bear E(−) or K(+) residues at positions $X_1 - X_4$ ($2^4 = 16$ compounds) was prepared according to the reported method (Figure 1).[10e] In this screening, we examined mixtures of two different peptides selected from entries 2–5 in Figure 1 together with an additional peptide, KKKK (16). This combination (two −4-peptides and one +8-peptide) was chosen for three reasons: 1) no species can form the fibrils by itself;[10e] 2) no two peptides selected from those listed can form a complementary pair for the heterogeneous assembly;[10e] 3) an equimolar mixture of these three species has a neutral net charge. Thus, six possible combinations of triplets were generated (Figure 2 a).

Three species were mixed in equimolar amounts (4 μM each) and incubated in a buffer (pH 7.4) for 5 days at 25 °C. The fibril formation of the triplets was then examined (Figure 2). A quantitative analysis of amyloid fibril formation was carried out by using an amyloid-specific dye, thioflavin T (ThT). This dye

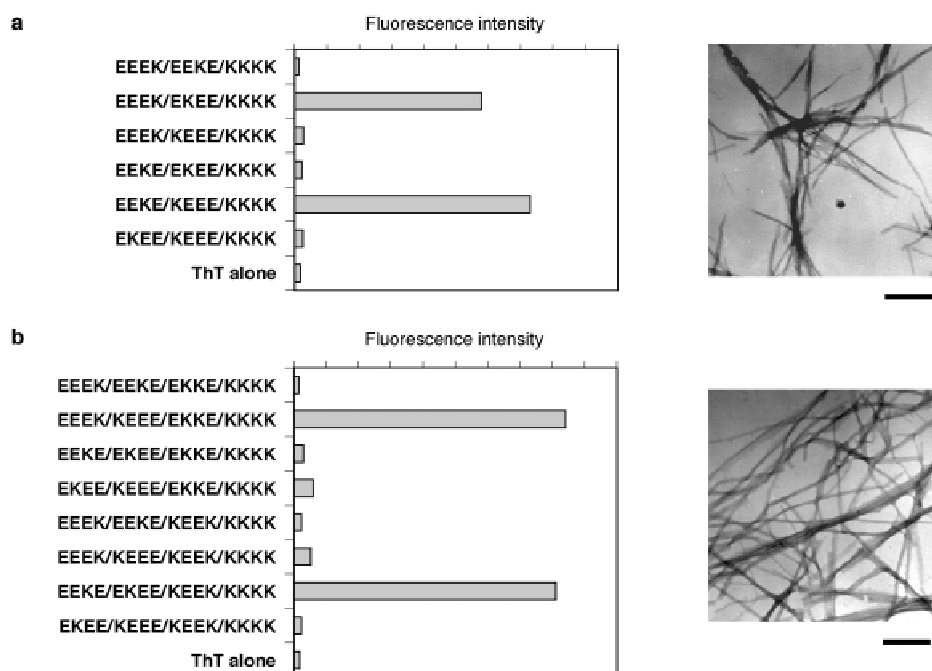

Figure 2. Fibril formation by three or four complementary peptide species. a) Left: ThT binding analysis of the three-peptide mixtures (4 μM each peptide, incubated in the buffer (pH 7.4) at 25 °C for 5 days); right: TEM image of the negatively stained fibrils formed by the EEEK/EKEE/KKKK mixture after 5 days incubation. b) Left: ThT binding analysis of the four-peptide mixtures (3 μM each peptide, incubated in the buffer (pH 7.4) at 25 °C for 5 days); right: TEM image of the negatively stained fibrils formed by the EEEK/KEEE/EKKE/KKKK mixture after 5 days incubation. The scale bars are each 200 nm.

associates with amyloid fibrils to cause a significant increase in fluorescence, which is dependent on the quantity of fibrils.[12] The results of this screening indicated that two combinations, EEEK/EKEE/KKKK and EEKE/KEEE/KKKK, are fibril-forming complementary triplets (Figure 2a). The fluorescence spectra of ThT after incubation in the presence of these three-peptide mixtures showed a new excitation maximum at around 440 nm and an enormous enhancement in emission at about 480 nm, both of which are characteristic of ThT bound to amyloid fibrils.[12] As a control experiment, ThT binding analyses of the complementary triplet EEEK/EKEE/KKKK were carried out (Figure 3a). The peptide solutions (12 μM total peptide), which contained all or some of the constituent species of the triplet, were incubated for 5 days at 25 °C. ThT was then added and the fluorescence intensity was measured. Significant enhancement in the fluorescence intensity of ThT was observed only in the presence of the triplet mixture and not when the mixture lacked any constituent peptide species, which suggests that all species are essential for assembly of the fibrils. Amyloid fibril formation by these triplet mixtures was confirmed by transmission electron microscopy (TEM; Figure 2a). The morphology of the fibrils formed by the triplet mixtures was similar to that of fibrils from naturally occurring amyloidogenic proteins.[13] The diameter of the fundamental fibrils ranges from 10 to 15 nm but some fibrils are bundled to form thicker fibers.

Circular dichroism (CD) studies revealed that the mixtures EEEK/EKEE/KKKK and EEKE/KEEE/KKKK initially form an α helix structure and then change to a β sheet structure within 2 days (Figure 4a). Furthermore, the α-helical nature of these three-peptide mixtures at the initial stage was higher than that of solutions that contained the single species (Figure 4a, dashed line), which suggests that these species interact with each other prior to β-sheet fibril formation (difference in

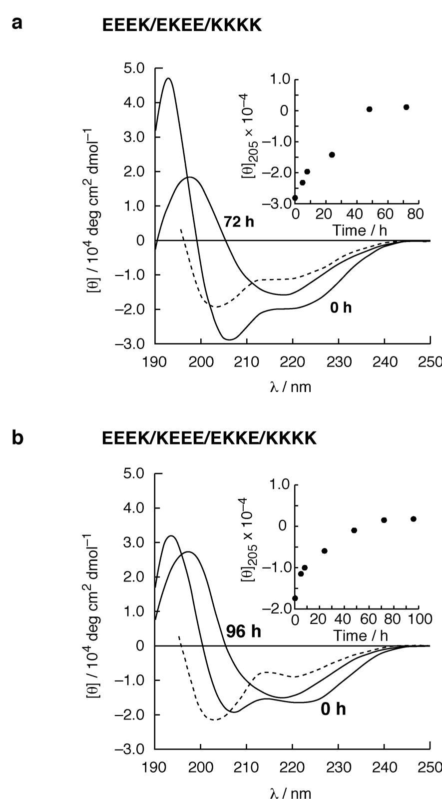

Figure 4. *Time-dependent CD spectral changes of the complementary three-peptide mixture (4 μM each peptide) EEEK/EKEE/KKKK (a), and the complementary four-peptide mixture (3 μM each) EEEK/KEEE/EKKE/KKKK (b). The three or four peptides were mixed and incubated in tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (20 mM, pH 7.4)/2.5 % trifluoroethanol (TFE) at 25 °C. The time course of [θ] at 205 nm is shown in the inset plots. CD spectra shown in a dashed line show the sum of the spectra of each species alone at 0 h. The CD profiles of the triplet EEKE/KEEE/KKKK and the quadruplet EEKE/EKEE/KEEK/KKKK (not shown) were similar to those shown.*

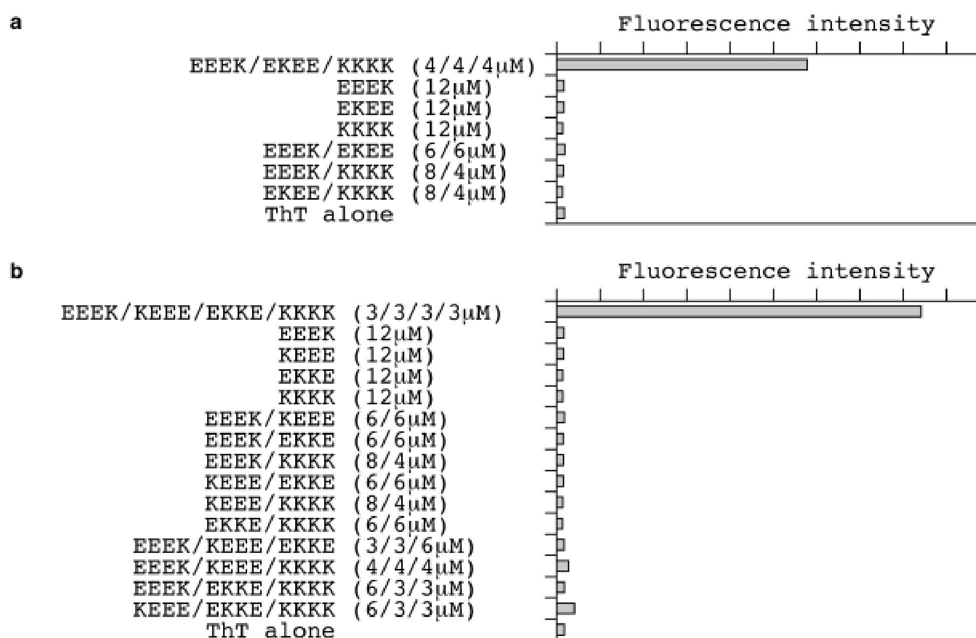

Figure 3. *Control ThT binding analyses for the complementary triplet EEEK/EKEE/KKKK (a) and the quadruplet EEEK/KEEE/EKKE/KKKK (b). The peptide solutions (12 μM total peptide), which contained all or some of the constituent species of the triplet or quadruplet, were incubated for 5 days at 25 °C, after which ThT was added and the fluorescence intensity was measured.*

$[\theta]_{222}$ between the spectrum of the mixture and the sum of the spectra for all the individual species: EEEK/EKEE/KKKK, 7100; EEKE/KEEE/KKKK, 7900 deg cm^2 dmol^{-1}). Similar enhancement of CD was observed in the case of complementary two-peptide pairings.[10e] The complementary interactions of the charged groups are also important in α helix formation at the initial stage.

Amyloid fibrils from four peptide species

Next, we attempted the complementary assembly of four peptide species into amyloid fibrils. In identifying complementary triplets, we found that the triplets EEEK/EEKE/KKKK, EEEK/KEEE/KKKK, EEKE/EKEE/KKKK, and EKEE/KEEE/KKKK were unable to assemble into fibrils (Figure 2a). We had the idea that the addition of a fourth species to these noncomplementary triplets might lead to the complementary assembly of four species into amyloid fibrils. The peptides EKKE and KEEK were chosen to be the fourth species because each is neutrally charged and is unable to form the fibrils individually.[10e] An equimolar mixture of four species, which include EKKE or KEEK, has a neutral net charge (two -4-peptides, one $+8$-peptide, and one ± 0-peptide). All eight possible combinations of quadruplets were generated (Figure 2b).

The four species were mixed in equimolar amounts (3 μM each) and incubated in buffer (pH 7.4) for 5 days at 25 °C. The fibril formation was then examined as described above. The ThT binding analysis indicated that two quadruplets, EEEK/KEEE/EKKE/KKKK and EEKE/EKEE/KEEK/KKKK, possess an outstanding ability to form amyloid fibrils but the others possess little or no such ability (Figure 2b). Control ThT binding analyses for the complementary quadruplet EEEK/KEEE/EKKE/KKKK suggested again that all species are essential to the assembly of fibrils (Figure 3b). The TEM study revealed that the morphology of the fibrils formed by the quadruplet mixtures is essentially identical to that of fibrils formed by the complementary triplet mixtures (Figure 2b).

CD studies showed that these quadruplet mixtures gradually underwent the structural transition from an α helix to a β sheet within 3 days (Figure 4b). The α-helical nature of these four-peptide mixtures at the initial stage was higher than that of solutions that contained the single species (Figure 4b, dashed line). The same observation was also made for the three-peptide mixtures and suggests that these species interact with each other prior to β-sheet fibril formation (difference in $[\theta]_{222}$ between the spectrum of the mixture and the sum of the spectra for all the individual species: EEEK/KEEE/EKKE/KKKK, 7500; EEKE/EKEE/KEEK/KKKK, 6200 deg cm^2 dmol^{-1}).

Complementary assembly model

The heterogeneous assembly of three or four species is illustrated by a schematic representation in Figure 5. An antiparallel molecular arrangement was applied to construct a model of the complementary assembly of triplet or quadruplet peptides. In this model, not only the charged face of the β strand but also the hydrophobic face opposite contributes to the complementary assembly. This model of amyloid assembly

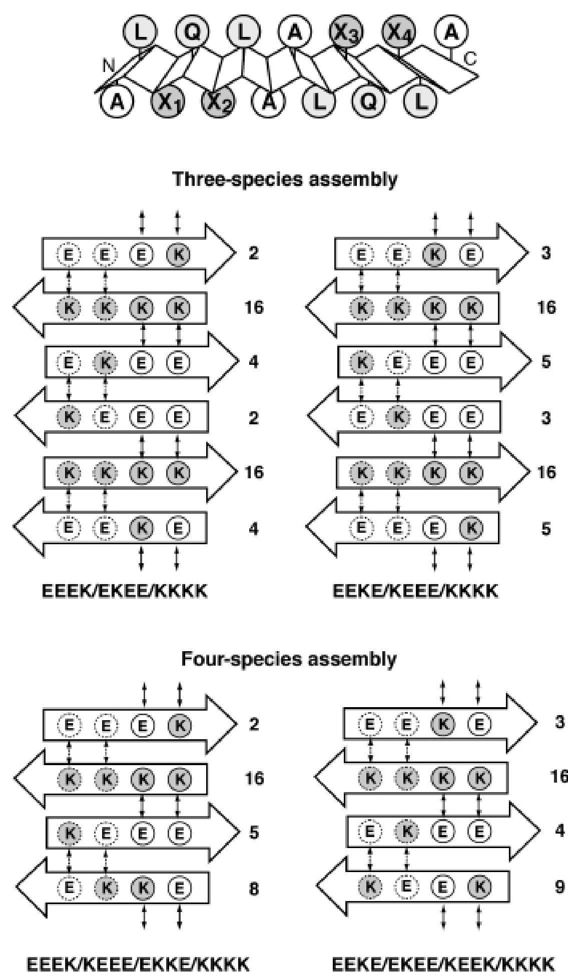

Figure 5. The lateral model for the complementary assembly of three (EEEK/EKEE/KKKK and EEKE/KEEE/KKKK) or four (EEEK/KEEE/EKKE/KKKK and EEKE/EKEE/KEEK/KKKK) peptide species as a laminated amyloid β sheet. The β strands are arranged in an antiparallel manner to maximize the charge interactions. The residues in solid circles denote those on the front side of β strand, while the residues in dashed circles denote those on the back side. The hydrophobic faces, which consist of L-Q-L sequences, are located on the side opposite the charged residues. The numbers refer to the entries in Figure 1.

combines both the complementary interaction of the electrostatic faces[10e] and the contribution of the hydrophobic faces.[10d] These model studies demonstrated that both the peptides that assemble homogeneously into amyloid fibrils and the peptide pairs that assemble heterogeneously must follow the rule of complementary pairing of charged residues E($-$) and K($+$) between β strands laminated into amyloid fibrils. In other words, peptides unable to form these electrostatic pairings cannot form amyloid fibrils. Also, with respect to the hydrophobic face of the β strand, similar complementary pairing was found to be essential to form two-species assemblies of analogous peptides mutated at the hydrophobic face.[10d]

The model in Figure 5 can explain why only two triplets or quadruplets from six or eight possible combinations of three or four peptides, respectively, are able to form amyloid fibrils. In the model constructs of these two triplets or quadruplets, complementary charged pairings at both front and back faces are

accomplished alternately in the β-sheet structure. The combinations that can form successive electrostatic pairings are these two particular triplets or quadruplets. In adjacent β strands, however, three of four charged groups in a strand are complementary to those in another strand. Therefore, these two triplets or quadruplets may need a much longer time (2 or 3 days) to finish the transition and to form amyloid fibrils than the peptides that assemble homogeneously and the two heterogeneous peptide pairs (3 – 4 h).[10] In contrast to the lateral model, a vertical model was also constructed (Figure 6). In this

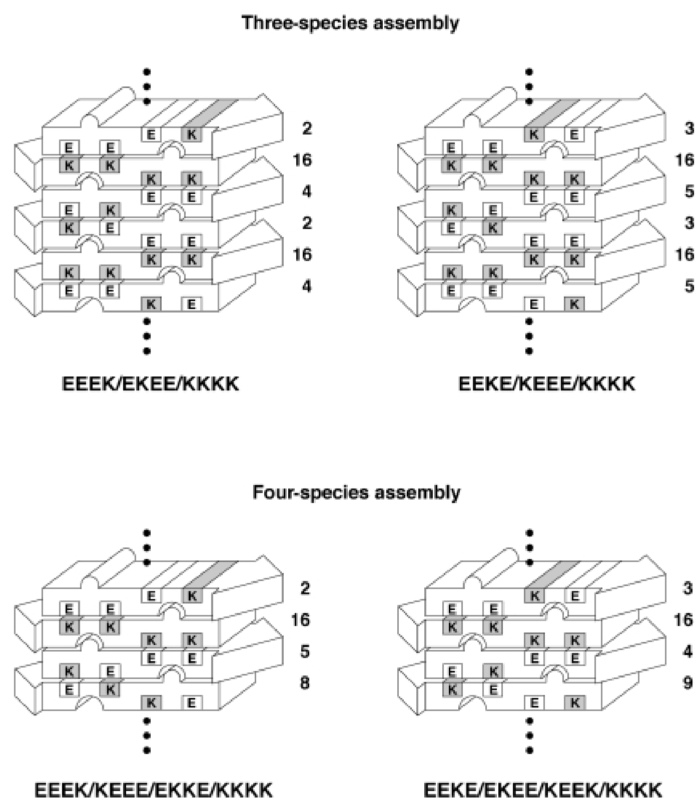

Figure 6. The vertical model of the complementary assembly of three (EEEK/EKEE/KKKK and EEKE/KEEE/KKKK) or four (EEEK/KEEE/EKKE/KKKK and EEKE/EKEE/KEEK/KKKK) peptide species. The interactions between the hydrophobic faces, which consist of L-Q-L sequences, are represented by projections and hollows.[10d] Interactions between side chains are accomplished vertically between β strands. The numbers refer to the entries in Figure 1.

model, both the electrostatic and hydrophobic interactions were complementarily arranged at both upper and lower faces of the β strands. The charged and hydrophobic side chains of the amino acid residues interact in a vertical fashion, which can be considered in the same manner as the interaction between β sheets laminated as shown in Figure 5.[10e] In the vertical model, complete sets of matched charged residues, E and K, are accomplished only when the two triplets or quadruplets are aligned in the manner shown in Figure 6. In either model, the heterogeneous assembly into fibrils is clearly accomplished by complementary interactions between two,[10e] three, or four peptide species, though further studies that examine the mechanism and precise molecular arrangement underlying the multiple peptide assembly are needed in future work. For example, the possibility that the mixed fibrils formed from the different peptides are each composed of a single species cannot be excluded at present. Although the orientation of the two peptide chains within a molecule is unknown and must be elucidated to afford a more precise model, it should be noted that the α-helical form of the two peptides at the initial stage facilitates the α to β transition and amyloid formation.[10a]

Conclusions

In this study we demonstrated that heterogeneous combinations of three or four peptide species each of which is unable to self-assemble individually ungero complementary assembly into amyloid fibrils and a simultaneous α to β structural transition. MacPhee and Dobson reported the incorporation of irregularly distributed fluorescent labels into fibrils by the co-assembly of two peptide species derived from natural proteins (1 % w/w of a labeled peptide and an unlabeled one).[8] The results presented here are the first demonstration of an approach to the construction of a heterogeneously assembled polypeptide fibril composed of multiple species in which the alignment and orientation of each species may be highly ordered. This ordered structure was accomplished by using simplified de novo designed polypeptides. Thus, this approach will enable the site-specific incorporation of multiple kinds of functional groups or structural probes (e.g. isotopes, fluorophores, and spin labels) into the polypeptide in a sequential array within the fibril. This is an important step toward the creation of nanoscale materials through the use of polypeptide fibrils with novel functional, physicochemical, and mechanical properties.[5–9] Moreover, the complementary assembly of β strands demonstrated here will lead studies toward the identification of the precise molecular details of the amyloid structure, which have not yet been fully resolved.[1–4] This strategy, when used with homologous peptides, can also be applied to the design of inhibitors developed on the basis of molecular models.

Experimental Section

Peptides: Peptides were synthesized by the solid-phase method by using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy[10] and the intermolecular disulfide bond was formed in 10% dimethyl sulfoxide/trifluoroacetic acid solution.[14] The obtained peptides were purified by reversed-phase HPLC and identified by MALDI-TOF MS and amino acid analysis. All peptide samples were dissolved in TFE as a stock solution to avoid the assembly of amyloid fibrils and then diluted with Tris-HCl buffer (20 mM, pH 7.4) for measurements. The final TFE content was 2.5%. The presence of 2.5% TFE slightly decreases the rate of the α to β transition and amyloid formation but the peptides showed a similar α to β transition profile to that observed in the absence of TFE.[10] Therefore, TFE (2.5%) has almost no effect on the measurements.

ThT binding analysis: Each peptide was dissolved in TFE (0.48 mM). Aliquots of the TFE solutions were mixed and the solution was

diluted with Tris-HCl buffer (20 mM, pH 7.4) to give 12 μM total peptide concentration and 2.5% TFE content. After incubation for 5 days at 25 °C, a ThT solution (240 μM in water) was added to the peptide solution and fluorescence measurements were carried out. Fluorescence intensities of ThT (20 μM) in the presence of the incubated peptides were recorded on a multi-well plate reader (excitation filter, 425 – 475 nm; emission filter, 525 – 535 nm) in a 96-well plate at 25 °C for the first screenings and the control experiments (Figures 2 and 3). The excitation and emission spectra were measured for ThT (6 μM) in the presence of peptides (12 μM) on a fluorescence spectrophotometer at 25 °C.

CD spectroscopy: Measurements were started immediately after dilution of the TFE solution with Tris-HCl buffer (20 mM, pH 7.4). The final concentration of peptides was 12 μM and the TFE content was 2.5%. The concentration of the peptide solutions was determined by quantitative amino acid analysis. CD spectra were measured at 25 °C by using a quartz cell with a 1.0 mm path length and recorded in terms of mean residual molar ellipticity ([θ] deg cm^2 d mol^{-1}). Turbidity of the solution, which disturbs the CD measurement, was not observed under these conditions.

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

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