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Stabilization of Peptide Fibrils by Hydrophobic Interaction

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Hydrophobic interactions play an important role in assembly processes in aqueous environments. In case of peptide amphiphiles, hydrophobicity is combined with hydrogen bonding to yield well-defined peptide-based aggregates. Here, we report a systematic study after the role of hydrophobic interactions on both stabilization and morphology of a peptide fibrillar assembly. For this purpose, alkyl tails were connected to a known β-sheet forming peptide with the sequence KTVIIE. The introduction of *n*-alkyl groups induced thermal stability to the assemblies without affecting the morphology of the peptide aggregates.

Introduction

The assembly behavior of peptides can be strongly influenced by the presence of amphiphilicity.[1] In principle, there are two methods for the introduction of amphiphilic character into peptides. First, a peptide can be built up out of a combination of hydrophobic and hydrophilic amino acids, either in alternating or block sequence.[2] A second well-studied method is based on the attachment of alkyl tails.[3−5]

In most cases, peptide amphiphiles assemble into fibrillar structures in solution. Even peptides that normally do not self-assemble yield stable aggregates upon attachment of alkyl chains.[6,7] A single glutamic acid can, for example, form fibrils when coupled to stearic acid.[8] These often well-defined aggregates are part of a broader class of self-assembling nanofibers based on amphiphilic molecules.[9] Peptide amphiphile based fibrils that are formed via this spontaneous assembly process have proven to be very useful, for example, as scaffolds for cell growth or as template for the buildup of materials.[6,10] Not only self-assembly can be induced via the attachment of alkyl tails,[11−13] but it can also change (the orientation of) the secondary structure[14,15] stabilizing a peptide in its bioactive conformation.

Self-assembly of amphiphilic peptides and proteins is also important in biological processes; exposure of hydrophobic patches in proteins has been linked to amyloid formation.[16] The increase in solvent-exposed hydrophobic regions precedes insolubilization and aggregation of the protein. This spontaneous transition of soluble proteins into amyloid fibrils is a major cause of several disorders such as Alzheimer's disease[17,18] and diabetes type II.[19] Investigating and influencing this aggregation process, which is based on a combination of hydrophobic interactions and β-sheet forming peptide sequences, can contribute to the understanding of fibril formation and can possibly be useful for drug development.[20] Because of this biological relevance, much research has been devoted in recent years to study model peptides that self-assemble into β-sheet like structures.[21−23]

Introduction of alkyl tails has been shown to induce stability to soluble, well-ordered peptide aggregates. Remarkably, so far only coiled-coil peptides[15,24] and nonaggregating peptides[25,26] have been modified with hydrophobic moieties to stabilize aggregates. In the research presented in this paper, we were especially interested in the effect of alkyl modification on peptide assembly of a known β-sheet forming peptide. We would like to know whether extra hydrophobic interactions could influence the stability of the existing peptide aggregate without affecting the existing peptide conformation. As a model peptide, the hexapeptide Ac-KTVIIE-NH_2 (K = lysine, T = threonine, V =

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valine, I = isoleucine, E = glutamic acid), as designed by de la Paz et al.,[22] was chosen, which is known to mimic amyloid fibril forming proteins. The charged lysine and glutamic acid residues in the peptide direct the aggregation process. The aggregation of the peptide proceeds in two stages; the aggregation process can be followed using circular dichroism spectroscopy (CD spectroscopy). Freshly dissolved, the peptide has a random coil conformation; first, the dissolved peptide organizes in a β-sheet polymer and a typical β-sheet spectrum develops.[22] Second, these polymers aggregate in regular fibril structures. This model peptide is one of the smallest peptide sequences to form stable β-amyloid fibers at a wide pH range.[27] The small size and the ability to introduce alkyl tails at both N and C terminus without disturbing the peptide structure make this model peptide an ideal candidate to systematically vary both the length and the position of alkyl chains and to study in detail the effect on assembly in a concentration- and temperature-dependent manner.

Experimental Section

Methods and Materials. ^{1}H NMR spectra were measured on a Varian Inova 400 MHz nuclear magnetic resonance (NMR) spectrometer. MALDI-TOF spectra were measured on a Bruker Biflex II mass spectrometer, and 2,5-dihydroxybenzoic acid (DHB) was used as matrix. UV−vis measurements were performed on a Varian Cary 50 UV−vis spectrophotometer and all CD measurements were performed on a Jasco J-810 spectropolarimeter with Peltier temperature control. IR spectra were recorded on a Thermo Wattson IR300 spectrometer, fitted with a Harrick ATR unit. Transmission electron microscopy (TEM) was performed on a JEOL 1010 transmission microscope. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semiautomatic peptide synthesizer. Peptide couplings were followed to completion using the Kaiser test.[28] All reagents were obtained from common commercial sources and were used as received.

Synthesis of Peptide R_1-Lys-Thr-Val-Ile−Ile-Glu-NH_2 (1, 2, 5−9). The peptide was synthesized using standard 9-fluorenylmethyl carbamate (Fmoc) peptide synthesis on a Breipohl resin.[29,30] Peptide coupling was achieved using 3 equiv of Fmoc amino acid and diisopropylcarbodiimide (DIPCDI, 3.3 equiv) with *N*-hydroxy benzotriazole (HOBt, 3.6 equiv) in DMF. The resin was swollen in DMF for 20 min prior to use. The Fmoc group was removed using piperidine in DMF (20% v/v, three times, 6 min). After coupling of the final amino acid, the Fmoc-protected peptide on the resin was washed thoroughly with DMF, dichloromethane, methanol, and diethyl ether. The resin was dried in vacuum and was divided in batches.

The dry resin was swollen for 30 min in DMF. Subsequently, the Fmoc-protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Three equivalents of the aliphatic carboxylic acid was dissolved in dichloromethane and 3.6 equiv of HOBt (1 M in DMF) was added. After mixing, 3.3 equiv of neat DIPCDI was added. The solution was added to the resin and the resin was agitated for 18 h. After washing the resin with DMF, dichloromethane, methanol, and diethyl ether, the resin was dried in vacuo. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water (95/5, v/v) for 4 h. After precipitation in diethyl ether, the peptides were lyophilized from acetic acid. The peptides were characterized using ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry (Supporting Information).

Synthesis of Peptide R_1-Lys-Thr-Val-Ile−Ile-Glu-$NHC_{16}H_{33}$ (3, 4). The C-terminal *n*-hexadecylamine tail for peptides **3** and **4** was introduced through reductive amination of 4-(4-formyl-3-methoxyphenoxy)butyryl aminomethyl resin.[31] In 15 mL of a mixture of DMF and methanol (1/1, v/v), 10 mmol of *n*-hexadecylamine was dissolved. To this solution, 10 mmol of acetic acid, 10 mmol of $NaCNBH_3$, and 1.0 g of resin (1.0 mmol) were added. The mixture was stirred slowly and was heated to 80 °C for 2 h. Subsequently, the resin was washed thoroughly with methanol and dichloromethane. To the secondary amine formed on the resin, Fmoc-protected glutamic acid was coupled using standard Fmoc solid-phase methodology using 3 equiv of the protected amino acid dissolved in DMF, HOBt (1 M in DMF, 3.6 equiv), and DIPCDI (1 M in DMF, 3.3 equiv). The resin was washed using DMF, methanol, dichloromethane, and diethyl ether and was dried thoroughly. The loading of the dry resin was determined using an Fmoc-cleavage UV-assay and was 0.37 mmol g^{-1} (75% of theoretical yield).

With the resulting resin, the further amino acids were again coupled using the standard Fmoc solid-phase synthesis protocol. After coupling of the final amino acid, the Fmoc-protected peptide on the resin was washed thoroughly with DMF, dichloromethane, methanol, and diethyl ether. The resin was dried in vacuum and was divided in batches.

The dry resin was swollen for 30 min in DMF. Subsequently, the Fmoc-protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Acylation of the N-terminus was achieved by a standard peptide coupling of the corresponding *n*-alkylcarboxylic acid. The resin was washed using DMF, dichloromethane, methanol, and diethyl ether. After drying, the products were cleaved from the resin by treatment with trifluoroacetic acid (95%, water 5%) for 3 h. After cleavage, the peptides were purified by precipitation in diethyl ether. The peptides were characterized using ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry (Supporting Information).

Fibril Formation. Solutions of peptides with a concentration of approximately 1 mg mL^{-1} were prepared in a pH 2.5, 20 mM glycine-HCl buffer, in siliconized Eppendorf tubes. The buffer was filtered through a 0.2-μm filter prior to use. The samples were sonicated at 50 °C for 30 min. Not all peptides completely dissolved upon this treatment, and therefore all solutions were again filtered through a 0.2-μm GHP membrane filter, after which the concentration was determined as described below. The peptide solutions were incubated for 5 days to allow for fibers to form.

Dilution series were prepared from the filtrated 1 mg mL^{-1} solutions of peptide. After dilution, the samples were incubated overnight at room temperature.

Determination of Concentration. Acetylated peptide **1** was dissolved in 20 mM glycine−HCl buffer of pH 2.5 to a concentration of 1 mg mL^{-1} (0.74 mM) by sonication. From the clear solution, a dilution series was prepared. The UV absorption spectrum of the solutions was determined in a 1-mm quartz cell at 220 nm (ϵ/dm^3 mol^{-1} cm^{-1} 4430). The concentration of peptide amphiphile solutions was determined using this known absorption coefficient.

CD Spectroscopy. Samples were measured at 25 °C in a 1-mm quartz cell. Temperature-dependent CD measurements were performed in a temperature range from 20 to 80 °C. The temperature was decreased or increased at a speed of 3 °C per minute. The elipticity was followed at 222 nm at 0.5-nm intervals, while a full spectrum was measured every 10 °C.

IR Spectroscopy. Solutions of fibrils in water were lyophilized and the dry samples were compressed on the ATR crystal with a pressure of 0.5 Kg. Infrared spectra were recorded for 128 scans at 2 cm^{-1} resolution.

TEM. A carbon-coated grid was placed on a drop of peptide solution for 5 min. The grid was blotted, dried in vacuo, and shadowed with platinum under an angle of 45°.

Results and Discussion

Synthesis of Peptide Amphiphiles. Two procedures were followed to introduce the aliphatic groups at either the N- or

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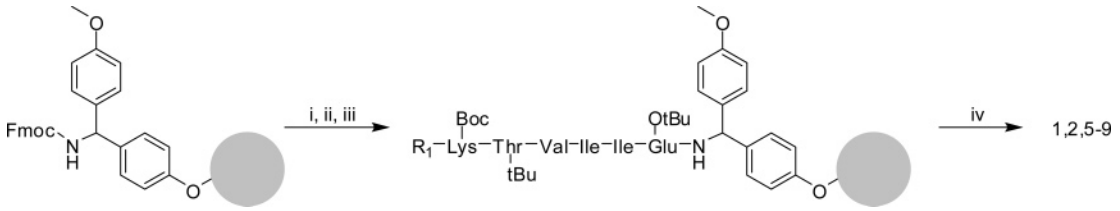
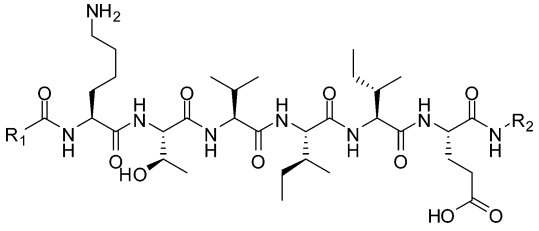


Figure 1. Solid-phase synthesis of N-terminally functionalized peptides **1**, **2**, and **5**−**9**. Reaction conditions: (i) piperidine (20% in DMF); (ii) Fmoc-Xxx, diisopropylcarbodiimide (DIPCDI), and *N*-hydroxy benzotriazole (HOBt) in DMF; (iii) $C_nH_{2n+1}COOH$, DIPCDI, and HOBt in dichloromethane; and (iv) TFA/H_2O (95/5).

Table 1. Peptides Synthesized



peptide	R_1	R_2
1	CH_3	H
2	$C_{15}H_{31}$	H
3	CH_3	$C_{16}H_{33}$
4	$C_{15}H_{31}$	$C_{16}H_{33}$
5	C_5H_{11}	H
6	C_7H_{15}	H
7	C_9H_{19}	H
8	$C_{11}H_{23}$	H
9	$C_{13}H_{27}$	H

C-terminus of the hexapeptide KTVIIE. In the first route, the aliphatic tails were incorporated at the N-terminus of the hexapeptide (Figure 1). The peptide was synthesized on a solid support using a Breipohl amide resin.[29,30] After the final amino acid was coupled, the alkyl chain could be introduced efficiently by a classical carbodiimide mediated coupling with the corresponding aliphatic carboxylic acid (Table 1, **1**, **2**, **5**−**9**). In the second route, the aliphatic moiety was introduced on the C-terminus of the peptide (Figure 2). For this purpose, an aldehyde functional resin was used and a coupling procedure was followed,[5,31,32] which was adapted from a method developed by Alsina et al. and Jensen et al.[33,34] A primary aliphatic amine was coupled to the aldehyde resin by means of a reductive amination, and the resulting secondary amine was subsequently the starting point for peptide synthesis (Table 1, **3**, **4**). The combination of these two highly efficient routes gives full access to independent functionalization of the C- and N-terminus, while keeping the advantages of solid-phase peptide synthesis. A series of peptide amphiphiles with varying alkyl chain length and position of the alkyl chain could therefore be readily prepared.

Peptide Amphiphile Assembly. Solutions of the peptides were prepared according to the following general procedure. Each peptide was dissolved in a 20 mM glycine−HCl buffer (pH 2.5) to a concentration of 1 mg mL^{-1}. The samples were sonicated at 50 °C for 30 min. Some peptides did not completely dissolve during the fibril preparation, therefore, all solutions were filtered. Subsequently, the samples were left to stand for 5 days at room temperature. UV−vis spectroscopy was used to determine the peptide concentration in the filtered solutions.

The first assembly parameter that was investigated was the effect of the position of the hydrophobic tail on stabilization of the fibrils. Alkyl tails were therefore introduced in three different ways: at the N-terminus, at the C-terminus, or both termini were functionalized simultaneously (peptides **2**, **3**, and **4**, respectively). Since we have shown previously that a strong stabilization of peptide aggregates was achieved via the introduction of a linear hydrophobic tail containing 16 carbon atoms,[7] hexadecanoic acid was introduced at the N-terminus or hexadecylamine at the C-terminus, or both were coupled to the same peptide. The aggregation properties of the model peptide KTVIIE (**1**)[22] were subsequently compared to these three amphiphilic peptides.

After filtration, the model peptide and the singly functionalized peptides were present in solution at a concentration of 0.6 mM. In contrast, peptide **4** with two introduced hydrophobic tails proved to be highly insoluble. No UV−vis or circular dichroism (CD) spectra could be recorded and no fibrils could be found using transmission electron microscopy (TEM). The two hydrophobic tails decrease the solubility of peptide **4** too much and structured aggregates cannot be formed anymore; therefore, this peptide was not examined any further.

The CD spectrum of the model peptide **1** showed a negative signal at 220 nm and a positive peak at 200 nm. This spectrum is typical for a β-sheet type assembly and is in accordance with previous reports.[22] TEM pictures furthermore confirmed the formation of fibrils. Similarly, the CD spectra of solutions of both singly modified peptides **2** and **3** show the same features, and TEM pictures showed the presence of fibrils (Figure 3), which possessed a similar morphology as the fibrils formed by the nonfunctionalized peptide. Peptide assembly was therefore not compromised by the introduction of the hydrophobic tails, which furthermore did not seem to promote nonspecific aggregation of the amphiphilic peptides.

Temperature-dependent CD measurements were deployed to investigate the extent of the stabilization of the fibril structure.[35] On increasing the temperature to 80 °C, the spectrum of the model peptide **1** changed to a minimum at 200 nm and a maximum at 220 nm (Figure 4). Such a spectrum is typical for a random coil conformation of this peptide. This change was not readily reversible upon cooling, indicating that fibril structures were completely disrupted. The β-sheet spectrum returned only slowly after several days at room temperature. In contrast, the CD spectra of both the C- and N-terminally modified peptides **2** and **3** did not change on increasing the temperature up to 90 °C (Figure 4), nor did subsequent cooling of the samples lead to any change in structure either. TEM showed that fibrils were still present in the solutions of the amphiphilic peptides after heating the sample, in accordance with the observed CD-signal.

As a second tool to probe the stability of the fibrils, the effect of peptide concentration on assembly behavior was also investigated for the series of peptides **1**−**3**. For this purpose, a

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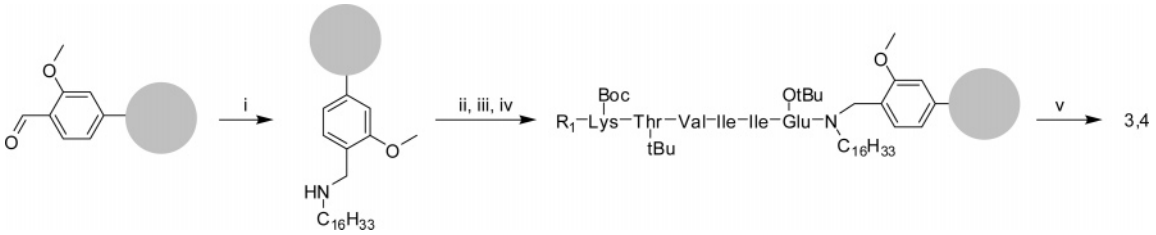

Figure 2. Solid-phase synthesis of C-terminally functionalized peptides **3** and **4**. Reaction conditions: (i) $C_{16}H_{33}NH_2$, acetic acid, and $NaCNBH_3$ in DMF/MeOH (1/1), 80 °C; (ii) Fmoc-Xxx, DIPCDI, and HOBt in DMF; (iii) piperidine (20% in DMF); (iv) $C_nH_{2n+1}COOH$, DIPCDI, and HOBt in dichloromethane; and (v) TFA/H_2O (95/5).

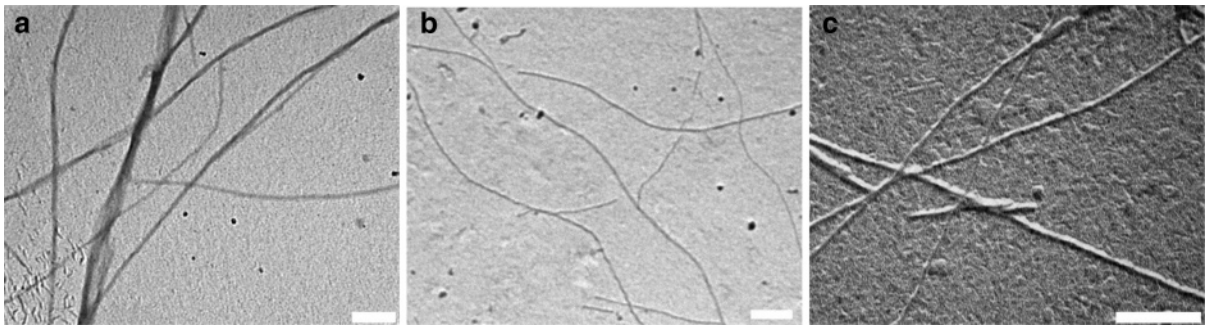

Figure 3. TEM pictures of Ac-KTVIIE **1** (a, bar represents 200 nm), C16-KTVIIE **2** (b, bar represents 200 nm), and Ac-KTVIIE-C16 **3** (c, bar represents 200 nm).

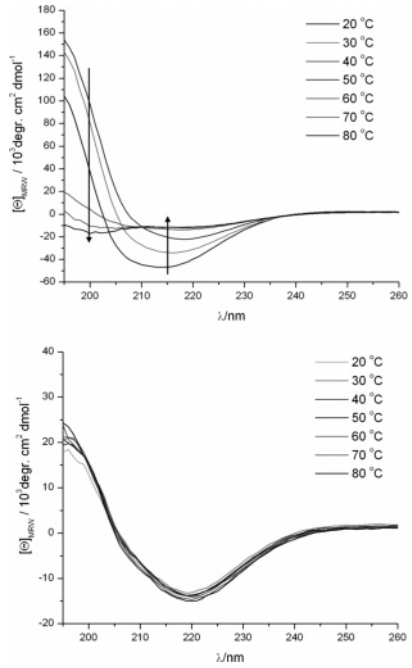

Figure 4. Temperature-dependent CD spectra of Ac-KTVIIE **1** (top) and C16-KTVIIE **2** (bottom), in the range of 20−80 °C. Arrows indicate the changes on temperature increase.

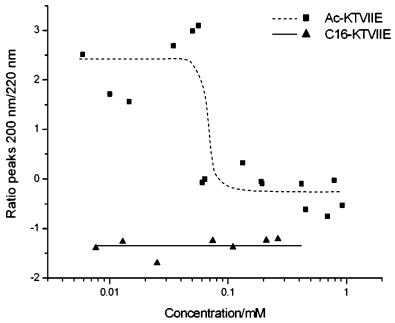

Figure 5. Concentration-dependent ratio of the peaks at 200−220 nm of peptides **1** and **2.** Lines are presented to guide the eye.

dilution series was made with glycine buffer, starting from a peptide concentration of approximately 1 mg mL^{-1}, which showed a minimum at 220 nm and a maximum at 200 nm in the CD spectrum. The diluted samples were equilibrated overnight at room temperature. The CD spectra were recorded in a 1-mm quartz cell, since the absorption of the used glycine buffer below 200 nm prohibited the use of a 1-cm cell.

Diluting the solution of nonstabilized peptide **1** caused a change of the CD-spectrum. At concentrations below 0.06 mM, the minimum at 220 nm in the CD spectrum disappeared and a minimum at 195 nm appeared. This indicates that also on dilution the fibrils were disassembled. In contrast, the CD spectrum of the amphiphilic peptides **2** and **3** did not change upon dilution; the minimum at 220 nm was present down to the lowest concentrations in which the CD signal could be measured accurately (Figure 5).

The structures that are formed by peptides **2** and **3** are stable to dilution or heating to 80 °C. To further examine the scope of the stabilization, a series of peptides was synthesized with decreasing amphiphilic character (Table 1). Since C-terminally and N-terminally functionalized peptides showed identical stabilization, only the N-functionalized series was prepared, because the latter peptides are synthetically more readily available.

The aggregation properties of peptides **5**−**9** at room temperature and at a concentration of 0.5 mg mL^{-1} were first examined. The CD spectra of these peptides showed a negative peak at 220 nm and a positive peak at 200 nm indicating the presence of peptide aggregates of a structure similar to peptides **2** and **3**. TEM pictures again showed the presence of fibrils with a morphology comparable to that of the model peptide.

To further probe the morphology, infrared spectra were recorded of lyophilized fibrils from aqueous solutions of peptides **1**, **2**, **5**−**9**. The amide I and II bands are indicative of the folding of peptides in aggregates.[36,37] All samples show a strong amide I band at 1625 cm^{-1} and the amide II band at 1535 cm^{-1} consistent with a parallel β-sheet conformation of the peptides.

The influence of the hydrophobic group on the temperature stability of the peptide fibrils was examined. On increasing the

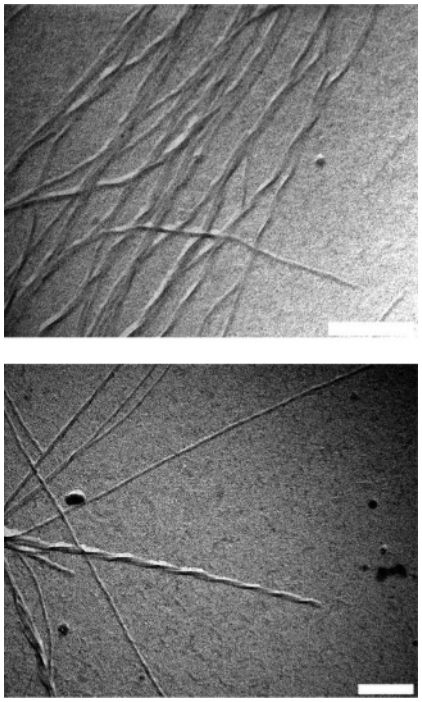

Figure 6. TEM pictures of fibrils formed by C8-KTVIIE **6** before (top, bar represents 200 nm) and after (bottom, bar represents 200 nm) heating to 80 °C.

temperature of a 0.6 mM solution of the C6 functionalized peptide **5**, the CD spectrum showed a marked decrease of the intensity of the peak at 220 nm. Keeping the sample at 80 °C, the peak at 220 nm disappeared whereas a peak at 190 nm became more intense, indicating a decrease in structure and hence the disassembly of the fibrils. Upon cooling, the peak at 220 nm did not return. Compared to model peptide **1**, the disappearance of the fibrils is, however, a slower process. The transition temperature of peptide **5** was 70 °C, whereas in case of the nonmodified peptide **1** already at 40 °C the process of disassembly could be observed.

The CD spectra of the C8 and C10 functionalized peptides **6** and **7** also changed upon heating. The intensity of the minimum at 220 nm and the maximum at 200 nm decreased; however, the CD-spectra did not change, suggesting the assemblies are at least partly intact. This was substantiated by the fact that for these aliphatic peptides the change was furthermore fully reversible; during cooling, the original CD-spectrum returned. Thus, although there is still some opportunity for rearrangement of the peptide backbone, the added hydrophobic interactions of the tails highly increase the stability of the aggregates. TEM pictures of the solutions showed fibrils, irrespective of thermal history (Figure 6).

The solution of C12 functionalized peptide **8** showed a stable CD spectrum in the whole examined temperature range. Expectedly, peptide **9** with the C14 tail also had a stable CD spectrum already observed for the C16 functionalized peptides **2** and **3**. The temperature measurements show that a small increase in hydrophobic interactions (going from C10 to C12) can already have a profound effect on the stability of peptide aggregates and peptide folding in the aggregate (Figure 7). Furthermore, the consistent character of the CD spectra within the series shows that the fibrils formed by the amphiphilic peptides all have a similar internal structure.

The concentration-dependent stability of aggregates of acylated peptides **5**−**9** was also determined using CD spectroscopy. On dilution, the CD spectrum of the C6 functionalized peptide **5** changed at concentrations below 0.04 mM. Compared to model peptide **1**, the transition took place over a broader concentration range and at a lower concentration. Similarly to the effect on the aggregate stability at increased temperatures, the hexyl tail was capable to a certain level of stabilization.

In contrast to the C6 tail, the aggregates of peptides with the longer tails did not disassemble upon dilution. The CD spectrum showed a distinct minimum at 220 nm down to the lowest concentration that could be measured, indicating that the peptide fibrils are stable at low micromolar concentrations.

On the basis of these results, we conclude that introduction of hydrophobic interactions to peptide **1** is an efficient method to stabilize aggregates formed by this peptide in glycine buffer. Remarkably, TEM and CD spectroscopy show that the morphology of the aggregates and the conformation of the peptide are not influenced by the introduction of the additional hydrophobic interactions. This strong morphological stability is independent of functionalization at either N- or C-terminus of the peptide. The peptide therefore controls the morphology of the aggregates, while introduction of alkyl tails only results in a higher stability of the fibrils to increased temperature and lower concentrations. Although the introduction of linear hydrocarbons with up to 10 carbon atoms already leads to remarkable added thermal stability, this induced stability seems to be maximal when the alkyl tail is C12 or longer. In contrast to previous examples where the systematic increase of hydrophobic interactions showed a steady increase in melting temperature[38] or a slow change in aggregate morphologies,[7] in our β-sheet forming peptide, added hydrophobic interactions seem to work as a switch without affecting the peptide conformation.

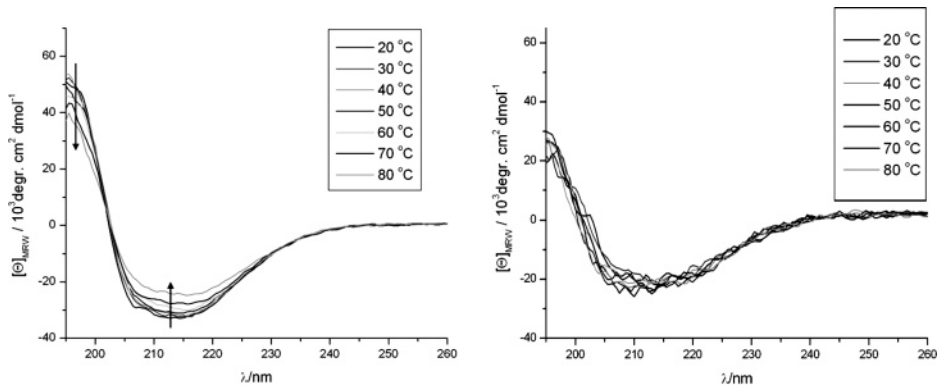

Figure 7. Temperature-dependent CD spectra of C8-KTVIIE **5** (left) and C12-KTVIIE **7** (right) in the range from 20 to 80 °C. Arrows indicate the change on temperature increase.

Conclusions

The introduction of a single alkyl tail at either the N-terminus or the C-terminus increases the stability of fibrillar aggregates formed by model peptide **1**. When a dodecyl (or longer) aliphatic chain is introduced, stability against heating and dilution of the peptide fibrils in an aqueous environment is obtained. To our knowledge, this is the first time that a detailed study of these types of amphiphilic peptides has been performed. Other techniques, such as X-ray diffraction, will have to give more information on the exact internal structure of the fibrils. Currently, we are further exploring the ability to switch the stability of peptide aggregates in situ by a controlled attachment and detachment of hydrophobic chains.

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Supporting Information Available: MALDI-TOF and ^{1}H NMR characterization, additional IR spectra, CD spectra, and TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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