Microwave-assisted click polymerization for the synthesis of Aβ(16–22) cyclic oligomers and their self-assembly into polymorphous aggregates†

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We report on the design, synthesis, and structural analysis of cyclic oligomers with an amyloidogenic peptide sequence as the repeating unit to obtain novel self-assembling bionanomaterials. The peptide was derived from the Alzheimer Aβ(16–22) sequence since its strong tendency to form antiparallel β-sheets ensured the formation of intermolecular hydrogen bridges on which the supramolecular assembly of the individual cyclic oligomers was based. The synthesis of the cyclic oligomers was performed *via* a microwave-assisted Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction of azido-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide as the monomer. The formation of cyclic oligomers, up to pentamers (35 amino acid residues), was verified by MALDI-TOF analysis and the individual cyclic monomer and dimer could be isolated by HPLC. Gelation behavior and the self-assembly of the linear monomer and the cyclic monomer and dimer were studied by TEM, FTIR and CD. Significant differences were observed in the morphology of the supramolecular aggregates of these three peptides that could be explained by alterations of the hydrogen bond network.

Introduction

The development of peptide-based polymers with unique properties like self-assembly has received much attention during recent years. This class of polymers has been used as drug delivery systems, medical devices, in tissue engineering, and for catalysis.[1] Since it has been shown that peptide-based polymers have better biocompatibility and bioavailability properties than their (semi)-synthetic congeners, novel applications of this class of biomaterials are still emerging. Of special interest are polymers that consist of relatively small building blocks with an intrinsic propensity to self-assemble into supramolecular constructs.[2]

A first prominent example of peptide-based supramolecular polymers has been given by Ghadiri *et al.*[3] They have found that cyclic peptides with alternating L- and D-amino acids form peptide nanotubes *via* hydrogen bond interactions of the peptide backbone. More recently, designed amphiphilic peptides and amyloidogenic peptide fragments have been used as the molecular self-recognition motif and, depending on the conditions (*e.g.* pH, bivalent metal ions, solvent), peptide nanotubes[4] or more fibrillar-like aggregates have been obtained as the supramolecular assemblies.[5] In this context, fragments of the highly amyloidogenic Aβ peptide, such as the dipeptide H-Phe-Phe-OH as described by Gazit and co-workers[6] and Görbitz,[4b–d] and the heptapeptide Ac-Lys-Leu-Val-Phe-Phe-Ala-Glu-NH_2 (Aβ(16–22)) as described by Lynn,[7] have been used to obtain peptide nanotubes. In the current literature several applications of peptide nanotubes or fibrillar aggregates as bionanomaterials, ranging from drug containers and nanoreactors to functional templates and biofilms, have been described.[2,8]

In previous studies we disclosed an attractive approach for the synthesis of peptide-based polymers.[9a,b] Bifunctional peptides, with an N-terminal azide moiety and a propargyl amide at the C-terminus, were polymerized *via* a Cu(I)-mediated click polymerization reaction.[10,11] Depending on the reaction conditions, either high molecular weight linear polymers or cyclic medium-sized oligomers could be obtained from the model dipeptide azido-Phe-Ala-propargyl amide. In the present study we describe another application of this click polymerization reaction, this time to synthesize cyclic oligomeric peptides in which the repeating motif is derived from the amyloidogenic Aβ(16–22) sequence, *i.e.* azido-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide (**1**, Fig. 1), to obtain a novel class of building blocks for the supramolecular assembly of peptide-based bionanomaterials. After running the click polymerization reaction, the isolated cyclic monomer and dimer have been individually used in a self-assembly process to explore their biomimetic organization behavior into peptide nanotubes and fibrillar aggregates and differences in morphology could be attributed to alteration of the intra- and intermolecular hydrogen bond network.

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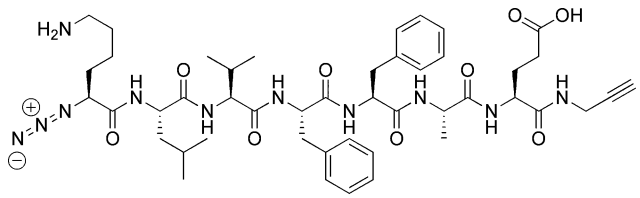


Fig. 1 Structural formula of the Aβ(16–22) peptide sequence, decorated with an N-terminal azido and a C-terminal alkyne moiety.

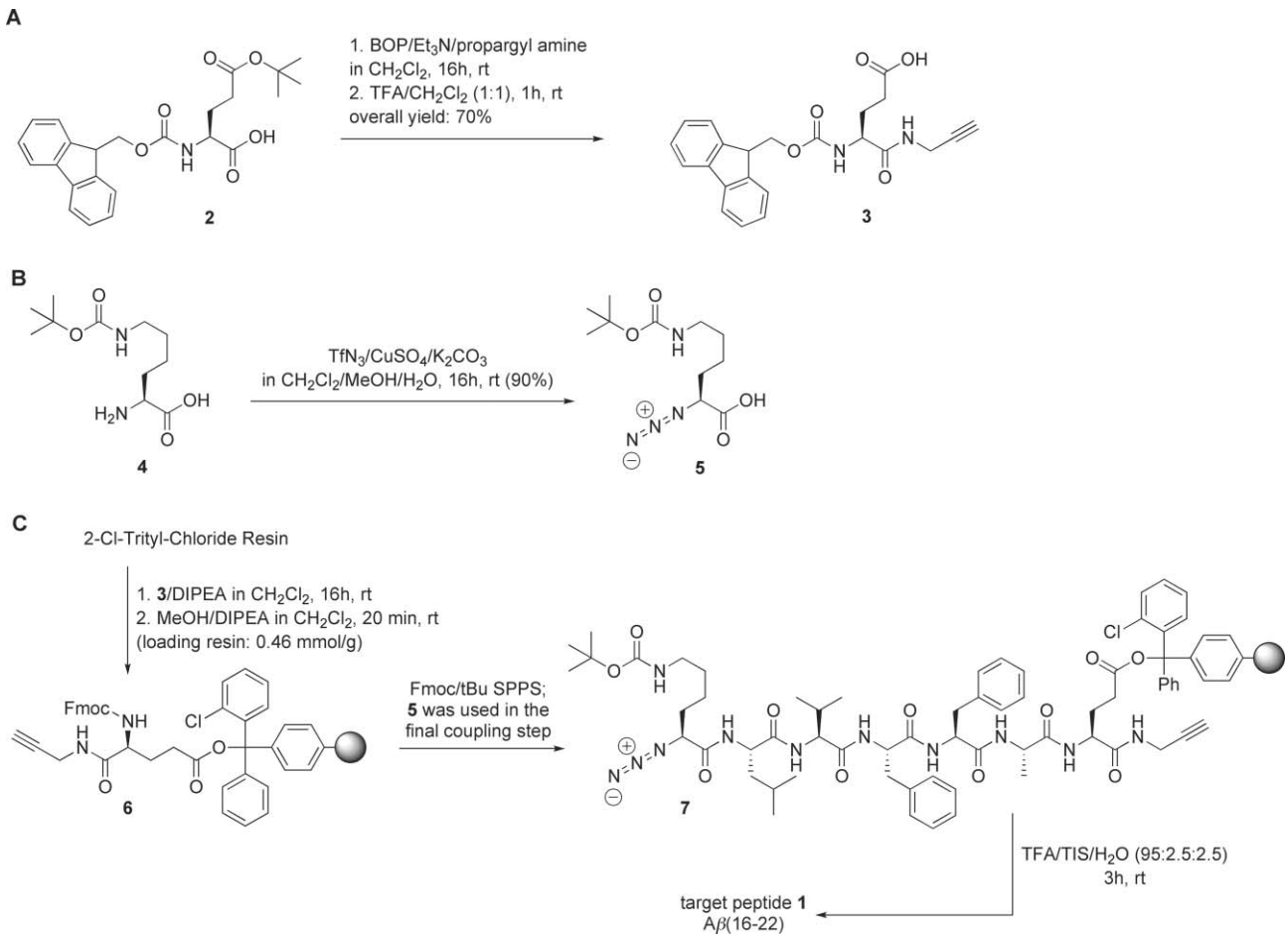
Results

Synthesis

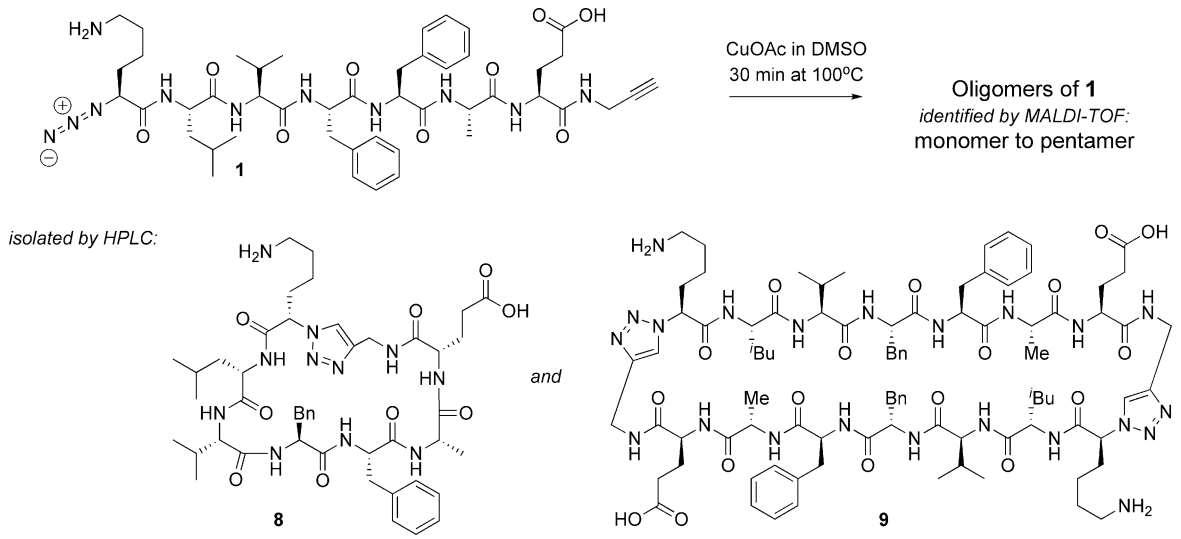
The synthesis of Aβ(16–22) peptide **1** and the required building blocks is depicted in Scheme 1. First, glutamic acid building block **3** and lysine derivative **5** were prepared starting from commercially available Fmoc-Glu(O^{t}Bu)-OH (**2**) and H-Lys(Boc)-OH (**4**), respectively. Fmoc-Glu(O^{t}Bu)-propargyl amide was obtained *via* a BOP-mediated coupling of **2** and propargyl amine. The fully protected intermediate was treated with TFA to remove the ^{t}Bu-functionality, and the desired building block **3** was obtained in 70% overall yield. The diazo-transfer reaction to convert **4** into azide **5** (90% yield) was performed essentially as described by Lundquist and Pelletier,[12] which was based on previous reports by Wong and co-workers.[13]

The synthesis of Aβ(16–22) peptide **1** was performed on the solid support. Since the C-terminus of this peptide is modified into the propargyl amide, building block **3** was connected to the resin *via* its γ-COOH side chain functionality and the 2-chlorotrityl chloride resin[14] (Barlos' resin) is ideally suited for this purpose. After loading the resin with Fmoc-derivative **3** (loading was determined[15] to be 0.46 mmol g^{-1}), the peptide sequence was synthesized by Fmoc/^{t}Bu chemistry.[16] After cleavage from the resin, peptide **1** was analyzed by HPLC and characterized by mass spectrometry. The presence of the azide moiety was confirmed by FTIR, since the characteristic N_3-stretch vibration was found at ν 2107 cm^{-1}.

Peptide Aβ(16–22) **1** was used as the monomer in the microwave-assisted click polymerization reaction (as shown in Scheme 2). The reaction conditions to obtain cyclic oligomers from bifunctional monomers, as recently described by van Dijk *et al.*,[9] were used: a low monomer concentration of 20 mg mL^{-1} and CuOAc as the catalytic species under microwave-irradiation for 30 min at 100 °C. Attempts to run the click reaction in DMF or DMF–H_2O mixtures failed, mainly due to the low solubility of **1**. Since the Aβ(16–22) peptide sequence has a high intrinsic tendency to



Scheme 1 Synthesis approach for the preparation of Aβ(16–22) peptide **1**. (A) Synthesis of glutamic acid building block **3**. (B) Synthesis of lysine building block **5**. (C) Solid phase synthesis of Aβ(16–22) peptide **1**.



Scheme 2 Microwave-assisted click polymerization of Aβ(16–22) peptide **1** and the structural formulas of the isolated cyclic peptides **8** and **9**.

aggregate, DMSO was used as an alternative solvent to disrupt any premature formation of secondary structure of the monomer, and the click reaction was run in this solvent with an increased amount (50 mol%) of CuOAc. The click polymerization reaction product was analyzed by MALDI-TOF mass spectrometry and revealed the presence of at least four different compounds (Fig. 2). Based on the molecular mass of the monomer ($[M + H]^+_{ave}$ 916.50), the observed peaks could be assigned to the cyclic dimer ($[M + H]^+_{ave}$ 1834.01), trimer ($[M + H]^+_{ave}$ 2750.47), tetramer ($[M + H]^+_{ave}$ 3666.94) and pentamer ($[M + H]^+_{ave}$ 4581.58). Purification by preparative HPLC resulted in the isolation of two compounds, characterized by a molecular mass of $[M + H]^+_{monoisotopic}$ 916.672 and $[M + H]^+_{monoisotopic}$ 1831.542, corresponding to the monomer and dimer, respectively. Based on FTIR analysis, it was concluded that the isolated compounds represented the cyclic monomer **8** (isolated yield: 15%) and cyclic dimer **9** (isolated yield: 33%), since the characteristic N_3-stretch vibration was absent. Although the formation of tri-, tetra- and pentamers was demonstrated by MALDI-TOF, their isolation by HPLC was unsuccessful.

The higher isolated yield of the cyclic dimer **9** compared to **8** might be explained by a mechanism that was proposed by Finn and co-workers[17] and which was corroborated by others.[18] This mechanism assumes that the favored dimerization involves the formation of a dialkyne–Cu(I) complex which rearranges *via* the energetically more favored *exo*-like conformation to give preferentially the cyclic dimer.[19] Furthermore, the 1,4-substituted triazole functionality has been described as a good β-turn inducing moiety,[20] thereby promoting the antiparallel organization of the newly formed dimer which ultimately results in the preferential formation of the cyclic dimer **9** (*vide infra*).

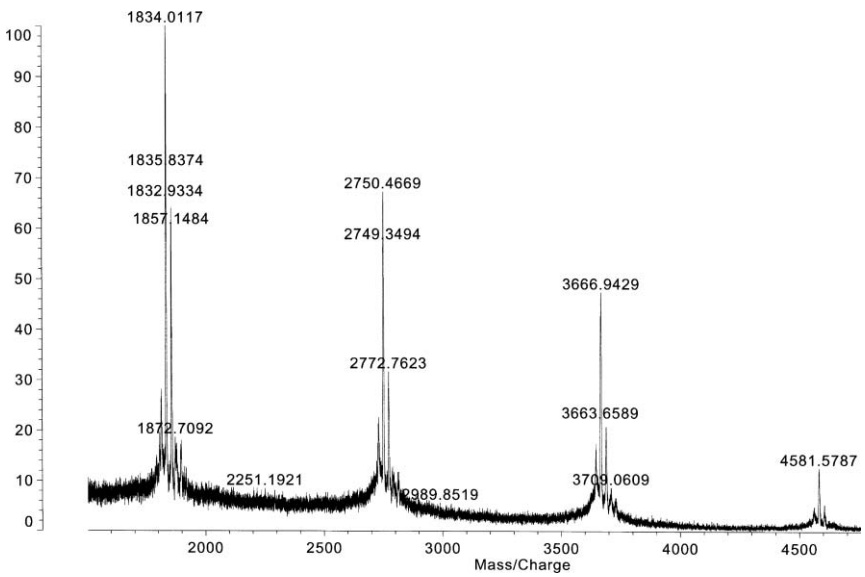


Fig. 2 Composition of the crude microwave-assisted click polymerization reaction product of peptide Aβ(16–22) **1**, as analyzed by MALDI-TOF spectrometry.

Supramolecular assembly

The supramolecular assembly of peptides **1**, **8** and **9** was studied by preparing a concentrated aqueous solution (6 mg mL^{-1} in H_2O–DMSO 9 : 1 v/v, pH 7) of each individual peptide. These samples were stored for three weeks at 4 °C prior to analysis. Gelation of the solution at sufficiently high concentration is a first indication of peptide aggregation and the formation of supramolecular assemblies. Although reference peptide **1** did not form a gel, analysis by TEM clearly indicated the presence of sheet-like lamellar assemblies (Fig. 3A). Analysis by FTIR[21] revealed, in addition to the azide and alkyne absorptions, the presence of a strong absorption at ν 1628 cm^{-1} of the peptide amide bond, indicative of aggregated antiparallel β-sheets with a lamellar supramolecular morphology. This result was in agreement with the X-ray powder diffraction data of Lynn and co-workers, who found that Aβ(16–22) formed supramolecular assemblies, with an antiparallel orientation of the assembled peptides.[7]

Likewise, cyclic peptide **8** did not form a gel, although broad fiber-like assemblies (up to 80 nm in width) were observed by TEM (Fig. 3B). However, the morphology of these assemblies was clearly different from that observed with peptide **1**. Moreover, FTIR analysis indicated that the amide I absorption was shifted from ν 1628 to 1672 cm^{-1}. This shift might be explained by assuming that self-assembly is largely based on hydrophobic interactions (Val/Leu), π–π interactions (Phe), and electrostatic interactions (Glu/Lys) rather than on intermolecular hydrogen bond formation *via* the peptide backbone. Additional evidence was obtained by CD spectroscopy which showed a positive maximum at λ 193 nm and a negative minimum at λ 205 and 235 nm of peptide **8** (Fig. 4), which implied an α-helical- rather than a β-sheet-like conformation.

The cyclic dimer **9** indeed formed a gel after three weeks of aging, and a dense network of fibrils was observed by TEM (Fig. 3C). Generally, these fibrillar entities were thinner and also longer than those formed by cyclic monomer **8**. In addition, the supramolecular assemblies as formed by **9** showed a high similarity with the amyloid fibrils formed by full length Aβ.[22] The formation of β-sheets was apparent by FTIR, from the presence of the characteristic absorptions at ν 1636 and 1686 cm^{-1} indicating an intra- and interstrand hydrogen bond pattern.[21] Moreover, CD spectroscopy showed a positive maximum at λ 195 nm and a negative minimum at λ 230 nm (Fig. 4), which is also a strong indicator of β-sheet formation.

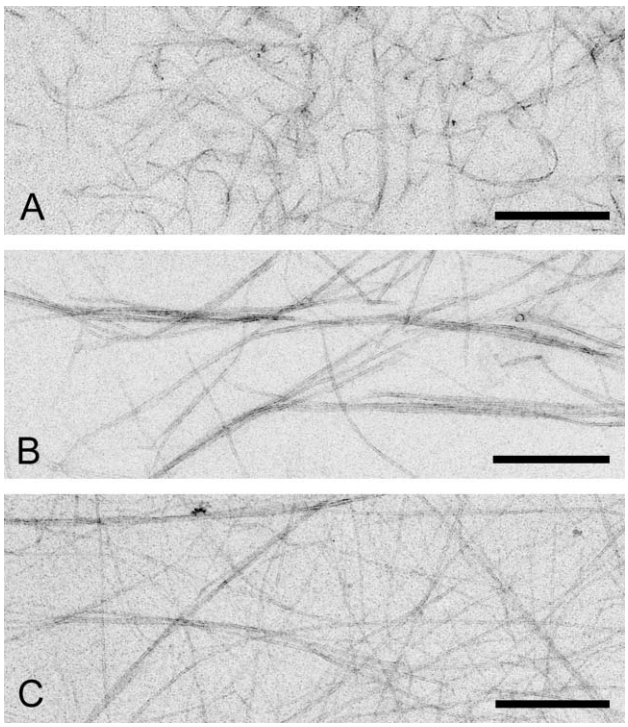


Fig. 3 TEM pictures of peptide **1** (A), cyclic peptide **8** (B), and cyclic dimer **9** (C). Scale bars represent 500 nm (conditions: 6 mg peptide/mL H_2O–DMSO 9 : 1 v/v, pH 7; peptide samples were aged for three weeks prior to analysis).

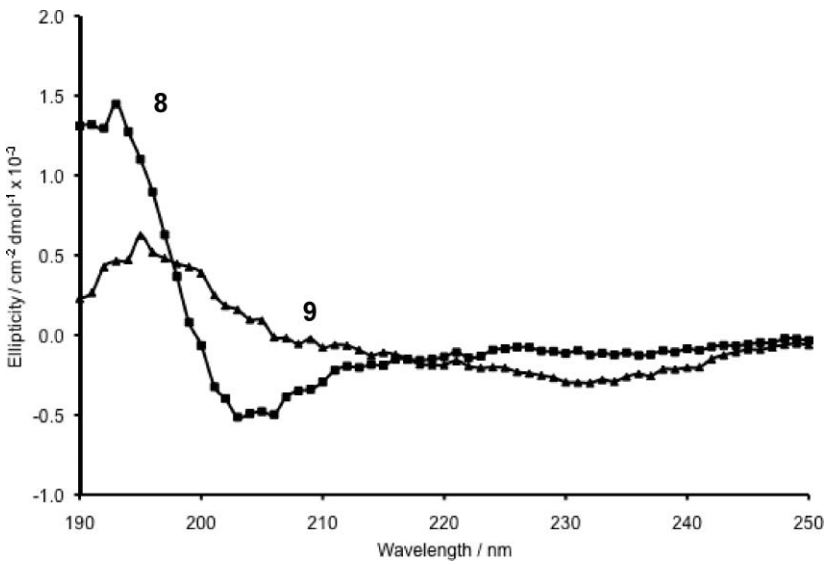


Fig. 4 CD spectra of cyclic peptide **8** (filled squares) and cyclic dimer **9** (filled triangles). Conditions: 0.3 mg peptide/mL H_2O–HFIP 9 : 1 v/v, pH 7; peptide samples were freshly prepared prior to analysis.

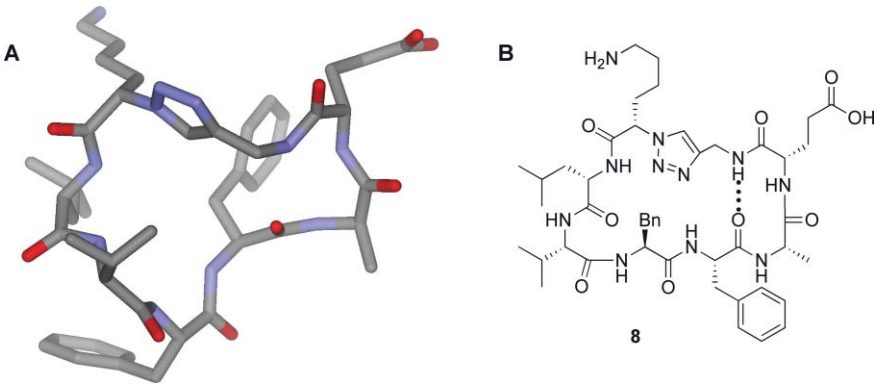


Fig. 5 (A) Lowest energy conformation of cyclic monomer **8**. (B) Structural formula of cyclic monomer **8** with the proposed intramolecular hydrogen bond indicated.

Discussion

The bifunctional heptapeptide azido-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide (**1**) was designed and synthesized as a novel monomer to be used in the microwave-assisted click polymerization approach toward the synthesis of peptide-triazoles as protein mimics. The choice of the monomer as the repeating peptide sequence is of crucial importance to obtain soft material-like properties,[23] such as those displayed by spider silk, elastin and collagen as the most prominent examples. The sequence of **1** corresponds to residues 16–22 from the highly amyloidogenic Aβ-peptide, which play a prominent role in Alzheimer's disease.[24] The intrinsic propensity of **1** to self-organize into well-defined nanostructures, as was shown by Lynn and co-workers,[7] enticed us to investigate the relationship between the molecular structure of **1** (linear, cyclic, or oligomeric) and the supramolecular folding morphology.

For a rapid access to cyclic oligomers of **1**, the microwave-assisted click polymerization approach as previously reported by us[9] was applied.[25,26] Although oligomers up to cyclic pentamers, *i.e.* 35 amino acid residues, were identified by MALDI-TOF analysis, the cyclic monomer **8** and cyclic dimer **9** were isolated as major components by preparative HPLC in a yield of 15 and 33%, respectively. The head-to-tail cyclodimerization was found to occur rapidly.[17-19] According to Finn and co-workers,[19] 'cyclodimerization depends on the ability of the azido–alkyne peptide to form in-frame hydrogen bonds between chains in order to place the reacting groups in close proximity and lower the entropic penalty for dimerization'. The FTIR analysis of cyclic dimer **9** is in agreement with this and characteristic absorptions at ν 1636 and 1686 cm^{-1} indicated an intra- and interstrand hydrogen bond pattern.[21] Moreover, CD spectroscopy also confirmed the presence of β-sheets.

Valéry *et al.* described the self-assembly of a cyclic octapeptide (lanreotide) and proposed a molecular structure of the formed nanotubes.[27] The β-hairpin structure of lanreotide together with the presence of hydrophobic aromatic side chains as in β-naphthylalanine, tyrosine and tryptophan, and hydrophilic side chains as in lysine and threonine, and a C-terminal carboxamide, strongly favored segregated π–π interactions and polar interactions in the self-assembly into peptide nanotubes. Fig. 5A shows an image of the lowest energy conformation of monocyclic peptide **8** in which a single intramolecular hydrogen bond is present (Fig. 5B). The calculated lowest energy conformation is in agreement with the FTIR data, indicating an intramolecular hydrogen bond (ν 1672 cm^{-1}). In addition, this conformation hints at an amphiphilic structure since the triazole moiety, as an amide bond mimic and hydrogen bond acceptor,[28] as well as the lysine and glutamic acid side chains are located on the same side of the molecule, while the hydrophobic side chains of leucine, valine, phenylalanine, and alanine are grouped on the other side of the molecule. Analogous to the model proposed by Valéry *et al.*,[27] monocyclic peptide **8** may form supramolecular assemblies as schematically shown in Fig. 6 in which self-assembly is driven by both electrostatic interactions of the oppositely charged

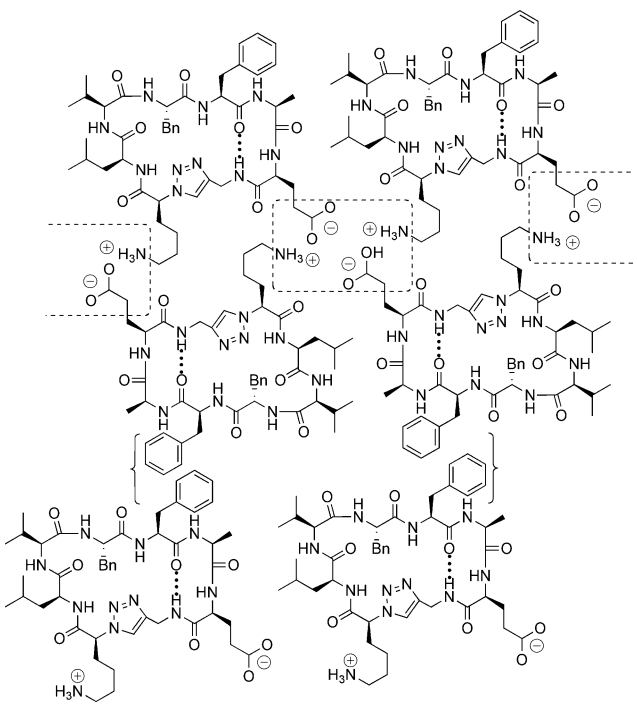


Fig. 6 Structural representation of the supramolecular assembly in the case of cyclic monomer peptide **8** (dotted lines: intramolecular hydrogen bridge, dashed box: electrostatic interactions, brackets: π–π and hydrophobic interactions).

Glu/Lys side chains and intermolecular π–π interactions of the phenylalanine side chains. Since the cyclic dimeric peptide **9** adopts an antiparallel β-sheet, as judged by FTIR and circular dichroism (Fig. 4), self-assembly might be due to hydrogen bond formation *via* the peptide backbone, as is schematically represented by Fig. 7.

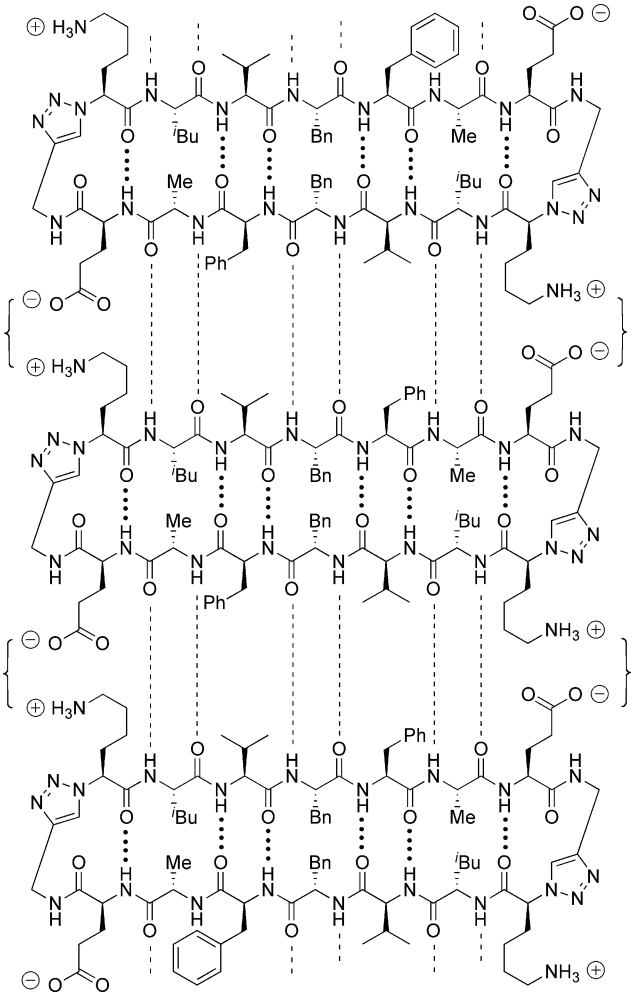


Fig. 7 Structural representation of a β-pleated sheet in the case of cyclic dimer peptide **9** (dotted lines: intramolecular hydrogen bridges, dashed lines: intermolecular hydrogen bridges, brackets: electrostatic interactions).

In summary, the facile solid phase synthesis of a bifunctional peptide derivative, azido-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide, of the amyloidogenic Aβ(16–22) fragment and its subsequent application as monomer in the microwave-assisted click polymerization to give cyclic oligomers have been described. Polymerization resulted in the formation of at least cyclic pentamers as judged by MALDI-TOF analysis. In addition, the cyclized monomer as well as the cyclic dimer could be isolated by preparative HPLC. Self-assembly of the cyclized monomer resulted in broad fiber-like aggregates while a dense network of amyloid fibrils was observed in the case of the cyclic dimer. This polymorphism of the supramolecular assemblies observed by electron microscopy might be attributed to a different conformation of the individual monomeric and dimeric peptides based on FTIR, CD and molecular dynamics calculations.

Since it has been shown in this study that the linear monomer, cyclic monomer and cyclic dimer differ in their self-assembly behavior, it is expected that controlling the molecular geometry of the peptide holds promise for directing self-assembly in a predictable way. This should ultimately lead to the development of designed nanostructured biological materials with a broad range of applications such as scaffolds for tissue-engineering and -repair, biocompatible coatings and films, templates for bone mineralization, novel biodegradable drug delivery systems, like nanocontainers, and stimuli-responsive hydro/organogels.[1,2,29]

Experimental

Chemicals, instruments and general methods

Chemicals were obtained from commercial sources and used without further purification. Peptide grade solvents used for solid phase peptide synthesis were stored on 4 Å molecular sieves. Microwave-assisted reactions were performed in a Biotage initiator apparatus with pressure and temperature control. ^{1}H-NMR spectra were recorded either on a Varian G-300 (300 MHz) or on a Varian Inova-500 (500 MHz) spectrometer and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C-NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shift values are given in ppm relative to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). The ^{13}C-NMR spectra were recorded using the attached proton test (APT) pulse sequence. Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat and are uncorrected. Optical rotations were measured on a Jasco P1010 polarimeter at ambient temperature. Column chromatography was performed with Silica-P Flash silica gel (Silicycle). Retention factor values (R_f) were determined with thin layer chromatography (TLC) by using Merck pre-coated silica gel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin, or Cl_2-*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane (TDM).[30] Preparative HPLC runs were carried out on an Applied Biosystems 400 Semi Automated HPLC System equipped with an Applied Biosystems 757 UV/VIS Absorbance Detector (λ = 214 nm). The crude lyophilized peptides were dissolved in a minimum amount of TFA and loaded onto an Alltech Prosphere C4 column (250 × 22 mm, particle size: 10 μm, pore size: 300 Å). The peptides were eluted with a flow rate of 10 mL min^{-1} using a linear gradient from 100% buffer A to 75% buffer B in 120 min (buffer A: 0.1% TFA in H_2O–CH_3CN 95 : 5 v/v; buffer B: 0.1% TFA in CH_3CN–H_2O 95 : 5 v/v). The retention time values (R_t) and purities of the peptides were evaluated by analytical HPLC on an Adsorbosphere XL C8 column (250 × 4.6 mm, particle size: 5 μm, pore size: 90 Å) at a flow rate of 1.0 mL min^{-1} using a linear gradient from 100% buffer A to 100% buffer B in 20 min (buffer A: 0.1% TFA in H_2O–CH_3CN 95 : 5 v/v; buffer B: 0.1% TFA in CH_3CN–H_2O 95 : 5 v/v). Peptides were characterized either by electrospray ionization mass spectrometry (EI-MS) on a Finnigan LCQ Deca XP Max apparatus operating in a positive ionization mode or by MALDI-TOF analysis which was performed on a Kratos Axima CFR apparatus. As the external reference, ACTH(18–39) ($[M + H]^+_{monoisotopic}$: 2465.1989), and as the matrix, α-cyano-4-hydroxy-cinnamic acid were used, respectively. The mass of each analog was measured and the observed monoisotopic $[M + H]^+$ value

was correlated with the calculated $[M + H]^+$ value. Transmission electron microscopy (TEM) was performed on a Jeol 1200 EX transmission electron microscope operating at 60 kV. A sample (10 μL) of a peptide gel/solution (6 mg mL^{-1} in DMSO–H_2O 1 : 9 v/v), which was aged for three weeks at 4 °C, was placed on a carbon-coated copper grid. After 15 min of incubation, the excess of peptide was removed by washing the copper grid on a drop of demi-water (this washing step was repeated five times). Finally, the samples were negatively stained by methylcellulose–uranyl acetate and dried in air prior to analysis. Fourier transform infrared spectra (FTIR) were measured on a BioRad FTS 6000 spectrophotometer. A sample (100 μL) of a peptide gel/solution (6 mg mL^{-1} in DMSO–H_2O 1 : 9 v/v), which was aged for three weeks at 4 °C, was lyophilized and subsequently resuspended in D_2O (150 μL) and lyophilized again. This treatment was repeated twice. The lyophilized peptide sample was mixed with KBr and pressed into a pellet. The optical chamber was flushed with dry nitrogen gas for 1 min before data collection started. The interferograms from 128 scans with a resolution of 2 cm^{-1} were averaged and corrected for H_2O and KBr. Circular dichroism (CD) spectra were measured on an OLIS RSM 1000 CD Spectrometer. A freshly prepared solution of the peptide (3 mg mL^{-1}) in HFIP was diluted with HFIP–H_2O (1 : 9 v/v) to a final concentration of 0.3 mg mL^{-1} and directly placed in a 2 mm cuvette in a thermostatted optical chamber (20 °C), which was continually flushed with dry nitrogen gas. The CD spectra were measured at 1 nm intervals in the range of 190–250 nm as the average of 10 scans using a spectral bandwidth of 1.0 nm. Calculations of the lowest energy conformation of the peptides were performed on a SiliconGraphics O_2 workstation with MacroModel 7.0 using the organic builder and the peptide builder in the grow mode.[31]

Peptide gelation experiments

Each peptide was dissolved in DMSO–H_2O (1 : 9 v/v) to obtain a final concentration of 6 mg mL^{-1}. The aggregation state was determined by eye at regular time intervals by tilting the test tube and checking if the solution still flowed. If no flow was observed, gelation was said to have taken place. The gelation experiments were performed at room temperature.

Syntheses

Azido-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide (1). A polystyrene resin, functionalized with a 2-chloro trityl chloride linker (1 g, initial loading: 1.0 mmol g^{-1}, 1.0 mmol), was loaded with Fmoc-Glu(OH)-propargyl amide **3** (1.22 g, 3.0 mmol, 3 equiv.) in dry CH_2Cl_2 (10 mL) in the presence of DIPEA (1.57 mL, 9.0 mmol, 3 equiv.) as base for 16 h at room temperature. After filtration, the resin was washed with CH_2Cl_2 (3 × 10 mL for 5 min each) and any unreacted trityl chloride was capped by treatment with a mixture of MeOH–CH_2Cl_2–DIPEA (2 : 17 : 1 v/v/v; 2 × 10 mL for 10 min each). After drying resin **6**, the amount of **3** coupled to the resin was calculated by an Fmoc-determination according to Meienhofer[15] and was found to be 0.46 mmol g^{-1}. The peptide sequence was synthesized by Fmoc/^{t}Bu SPPS protocols on a 0.25 mmol scale (540 mg resin). The N-terminal N_3-Lys(Boc)-OH **5** (136 mg, 0.50 mmol, 2 equiv.) was coupled with HBTU–HOBt–DIPEA (2 : 2 : 4 equiv.) in NMP (10 mL) for 2 h at room temperature. All coupling reactions were monitored by the Kaiser test.[32] Resin **7** was treated with TFA–TIS–H_2O (95 : 2.5 : 2.5 v/v/v) for 3 h at room temperature to deprotect and cleave the peptide from the resin, and the peptide was precipitated in MTBE–hexane (1 : 1 v/v) at –20 °C. The crude peptide was lyophilized from *tert*-BuOH–H_2O (1 : 1 v/v). The peptide was purified by HPLC and identified and characterized by mass spectrometry and NMR spectroscopy, respectively. Heptapeptide **1** was obtained in 74% yield (310 mg) after purification. R_t 20.3 min; ν_{max}/cm^{-1} 2108 (N_3), 1695 and 1628 (C=O), 1540; ^{1}H-NMR (300 MHz, DMSO-d_6) δ = 8.37–8.30 (m, 2H, amide NH propargyl (1H)/amide NH Val(1H)), 8.18 (d, J = 7.2 Hz, 1H, amide NH Ala), 8.11 (d, J = 8.0 Hz, 1H, amide NH Phe), 8.00–7.93 (m, 2H, amide NH Phe (1H)/amide NH Glu (1H)), 7.83 (d, J = 8.8 Hz, 1H, amide NH Leu), 7.24–7.18 (m, 10H, arom H Phe (2 × 5H)), 4.53 (m, 2H, αCH Phe (2 × 1H)), 4.38–4.23 (m, 3H, αCH Ala (1H)/αCH Val (1H)/αCH Glu (1H)), 4.08 (t, J = 7.8 Hz, 1H, αCH Leu), 3.85 (m, 2H, CH_2 propargyl), 3.77 (t, J = 6.9 Hz, 1H, αCH Lys), 3.12 (m, 1H, HC≡), 3.05–2.68 (double m, 4H, βCH_2 Phe (2 × 2H)), 2.75 (m, 2H, εCH_2 Lys), 2.22 (m, 2H, γCH_2 Glu), 1.86–1.73 (m, 3H, βCH_2 Leu (2H)/γCH Leu (1H)), 1.86–1.73 (m, 2H, βCH_2 Glu), 1.73–1.30 (m, 6H, βCH_2/γCH_2/δCH_2 Lys (3 × 2H)), 1.53 (m, 1H, βCH Val), 1.21 (d, J = 7.2 Hz, 3H, βCH_3 Ala), 0.84 (dd, 6H, γCH_3/γ′CH_3 Val (2 × 3H)), 0.71 (d, J = 4.4 Hz, 6H, δCH_3/δ′CH_3 Leu); ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ = 173.9, 171.9, 171.4, 170.8, 170.7, 170.6, 170.4, 169.1, 137.6, 129.1, 128.0, 126.2, 80.9, 73.0, 60.9, 57.5, 53.6, 53.4, 51.7, 51.1, 48.2, 37.5, 30.7, 30.4, 30.2, 28.0, 27.6, 26.6, 24.2, 23.1, 22.2, 21.5, 19.1, 18.0; MALDI-TOF $C_{46}H_{65}N_{11}O_9$ requires 915.50, found m/z $[M + H]^+_{monoisotopic}$ 916.67, $[M + H]^+_{ave}$ 918.19, $[M + Na]^+_{ave}$ 940.21.

Fmoc-Glu(OH)-propargyl amide (3). Fmoc-Glu(O^{t}Bu)-OH **2** (4.25 g, 10 mmol), propargylamine hydrochloride (915 mg, 10 mmol) and BOP (4.42 g, 10 mmol) were dissolved in CH_2Cl_2 (100 mL) and this solution was cooled on ice before Et_3N (4.18 mL, 30 mmol, 3 equiv.) was added. The obtained reaction mixture was stirred for 1 h at 0 °C followed by 16 h at room temperature. Then, the solvent was removed under reduced pressure and the residue was redissolved in EtOAc (150 mL). The organic solution was subsequently washed with 1 M $KHSO_4$ (2 × 75 mL), sat. aq. $NaHCO_3$ (2 × 75 mL) and brine (1 × 50 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The obtained reaction product was used without further purification in the next reaction step. Fmoc-Glu(O^{t}Bu)-propargyl amide was dissolved in CH_2Cl_2–TFA (100 mL 1 : 1 v/v) and the reaction mixture was stirred for 2 h at room temperature. After this period of stirring, the reaction mixture was concentrated *in vacuo* and the residue was coevaporated with CH_2Cl_2 to remove any residual TFA. Then, the residue was purified by column chromatography (eluent: CH_2Cl_2–MeOH 95 : 5 v/v) to give amide **3** as a white solid in 70% overall yield (2.8 g). Mp 151–154 °C; R_f 0.20 (CH_2Cl_2–MeOH 95 : 5 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$–CD_3OD 9 : 1 v/v) δ = 7.97 (broad s, 1H, amide NH), 7.78–7.30 (9H, m, urethane NH (1H)/arom H (8H)), 4.40 (m, 2H, CH_2 Fmoc), 4.23 (m, 2H, CH Fmoc (1H)/αCH Glu (1H)), 4.00 (s, 2H, CH_2 propargyl), 2.38 (m, 2H, γCH_2 Glu), 2.30 (m, 1H, HC≡), 2.10–1.86 (double m, 2 × 1H, βCH_2 Glu); ^{13}C-NMR (75.5 MHz, $CDCl_3$–CD_3OD 9 : 1 v/v) δ = 175.2, 171.6, 156.5, 143.4, 141.0, 127.5, 126.8, 124.7, 119.6, 78.7, 71.2, 66.7,

53.6, 46.8, 29.7, 28.6, 27.5; EI-TOF-LCMS $[M + H]^+$ $C_{23}H_{23}N_2O_5$ requires m/z 407.1607, found 407.1626.

Azido-Lys(Boc)-OH (5). This compound was synthesized according to the procedure of Lundquist and Pelletier,[12] starting with commercially available H-Lys(Boc)-OH (**4**) on a 12 mmol scale and compound **5** was obtained as a pale yellow oil in 90% yield. R_f 0.57 ($CHCl_3$–MeOH–AcOH 95 : 18 : 3 v/v/v); $[\alpha]_D^{25}$ −20.9 (*c* 1.04 $CHCl_3$) (lit.,[12] −19.0 (*c* 1.0 $CHCl_3$)); ^{1}H-NMR (300 MHz, $CDCl_3$) $\delta =$ 10.45 (broad s, 1H, COOH), 4.71 (m, 1H, urethane εNH), 3.91 (m, 1H, αCH), 3.13 (m, 2H, εCH_2), 1.84 (m, 2H, βCH_2), 1.52 (m, 4H, $\gamma CH_2/\delta CH_2$ (2 × 2H), 1.44 (s, 9H, $(CH_3)_3$ Boc); ^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta =$ 174.4, 156.3, 79.7, 61.8, 40.2, 30.9, 29.4, 28.4, 22.8.

Microwave-assisted polymerization reaction of N_3-Lys-Leu-Val-Phe-Phe-Ala-Glu-propargyl amide. Peptide monomer **1** (40 mg, 43.7 μmol) was dissolved in N_2-purged DMSO (2 mL) and CuOAc (3 mg, 24.6 μmol, 0.56 equiv.) was added. The reaction mixture was placed in the microwave reactor and irradiated for 30 min at 100 °C. The clear solution was transformed into a turbid gel. The gel was dissolved in an additional amount of DMSO (3 mL) and the solution was concentrated *in vacuo* (SpeedVac) to obtain a solid pellet. The greenish solid was redissolved in CH_3CN–H_2O (5 mL, 1 : 1 v/v) and lyophilized. After purification by HPLC, two off-white peptides could be isolated, identified and characterized by analytical HPLC, FTIR and MALDI-TOF analysis.[33]

Cyclic peptide (8). This peptide, the cyclic monomer, was isolated in 15% yield (6 mg, 6.6 μmol). R_t 19.6 min; ν_{max}/cm^{-1} 1672 (C=O), 1548; MALDI-TOF $C_{46}H_{65}N_{11}O_9$ requires 915.497, found m/z $[M + H]^+_{monoisotopic}$ 916.672, $[M + Na]^+_{monoisotopic}$ 938.627.

Cyclic peptide (9). This peptide, the cyclic dimer, was isolated in 33% yield (13 mg, 7.1 μmol). R_t 19.1 min; ν_{max}/cm^{-1} 1687 and 1635 (C=O), 1542; MALDI-TOF $C_{92}H_{130}N_{22}O_{18}$ requires 1830.994, found m/z $[M + H]^+_{monoisotopic}$ 1831.542.

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