Putting a spin on it: amyloid aggregation from oligomers to fibrils

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2 Synthesis and first CD and EPR characterization of small amyloid peptides designed to form oligomers

Electron Paramagnetic Resonance (EPR) is a powerful tool to study peptides. The use of 2,2,6,6-tetramethyl-N-oxyl-4-amino-4-carboxylic acid (TOAC) as a spin label that incorporates directly into the backbone of peptides, is advantageous to determine peptide backbone motion. In this study we report the synthesis of three TOAC peptides modeled after K11V (KVKVLGDVIEV), an amyloid peptide that self-aggregates to oligomers with a β-sheet configuration [Laganowsky, A. *et al., Science* **335,** 1228–1231 (2012)]. By Solid Phase Peptide Synthesis (SPPS), we prepared T0EZ with the TOAC (**Z**) at the N-terminus (**Z**KVKVLGDVIEVGG), T5EZ (KVKV**Z**GDVIEVG) and T12EZ (KVKVLGDVIEV**Z**G). We checked the aggregation propensity at room temperature in liquid solution by continuous-wave EPR and Circular Dichroism. Only T0EZ aggregates similarly to K11V; T5EZ and T12EZ do not. This suggests that TOAC at specific positions inhibits aggregation, implying that its position must be chosen carefully to avoid modifications in the properties of the peptide.

2.1 Introduction

Electron Paramagnetic Resonance (EPR) is increasingly used to understand the structure and behavior of proteins and peptides, see for example,[47,50,57–59] amongst which the difficult to study class of Intrinsically Disordered Proteins[60,61], for example in relation to amyloid aggregation.[61–66]

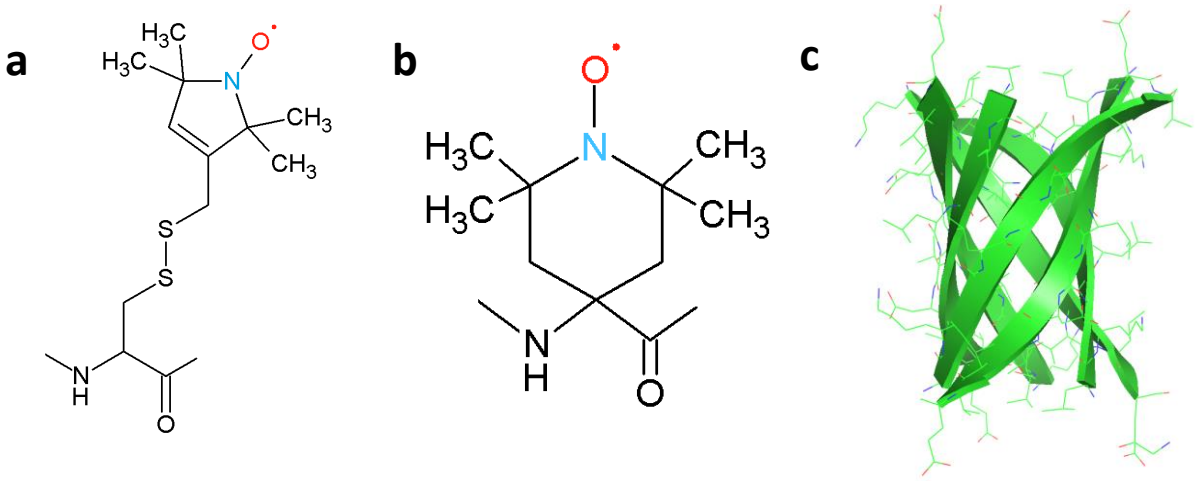

Figure 2.1 *(a) Chemical structure of MTSL attached to the Cys amino acid. (b) Chemical structure of the TOAC amino acid. (a) Ribbon configuration of the oligomer of K11V obtained by X-Ray crystallography*[67]

To apply EPR, the protein or peptide of interest has to be spin labelled. For globular and membrane proteins the site-directed spin-label method, in which a nitroxide spin label is attached via a cysteine to the protein backbone (see Figure 2.1a),[45,47,59,68,69] is almost universally applied. For small peptides, the intrinsic flexibility and the long linker length of these common spin labels can be problematic. Several more rigid spin labels were proposed.[70–74] For peptides, the backbone-fixed α-amino acid TOAC (Figure 2.1b), in which the motion of the nitroxide is closely coupled to the peptide backbone motion, is the most promising approach.[63,75–83] The TOAC label follows the peptide motion more faithfully than the cysteine-linked labels and therefore it would be better suited for studying the peptide behavior. Synthesis of TOAC labeled peptides can be performed by Solid Phase Peptide Synthesis (SPPS),[40,84–89] or organic synthesis methods.[76–81,90–93]

To test the suitability of TOAC in the study of amyloid-peptide aggregation, we chose the K11V peptide as a model. This peptide was shown to form stable oligomers as shown in Figure 2.1c

Here we describe the SPPS of three variants of the K11V peptide, containing the TOAC spin label in three different positions. The original K11V sequence was expanded with Glycines at the C-terminus to facilitate the synthesis, generating the “new” wild-type K13G.

To determine the aggregation propensity, we use liquid solution, room-temperature continuous-wave EPR (cw EPR). This method is sensitive to nano-second rotational motion of the nitroxide and detects aggregation by broadening of the lines of the spectrum.[62,90–93] Also Circular Dichroism (CD) is sensitive to aggregation, because the monomers convert from random coil to β-sheet oligomers (for more details, see SI). Both forms have a characteristic CD signature.[94]

In this chapter we report the synthesis and initial characterization of the TOAC peptides. We show that the peptide with an N-terminal TOAC aggregates similarly to the wild type, whereas the other two do not aggregate.

2.2 Results

Table 2.1 *Sequences of the peptides used and aggregation properties. TOAC (2,2,6,6-tetramethyl-N-oxyl-4-amino-4-carboxylic acid)**:** position 0 for T0EZ, position 5 for T5EZ, position 12 for T12EZ. Aggregation determined by CD and EPR.*

peptide	sequence														aggregation by CD	aggregation by EPR
K11V		K	V	K	V	L	G	D	V	I	E	V			-	-
K13G		K	V	K	V	L	G	D	V	I	E	V	G	G	YES	-
T0EZ	TOAC	K	V	K	V	L	G	D	V	I	E	V	G	G	YES	YES
T5EZ		K	V	K	V	TOAC	G	D	V	I	E	V	G		NO	NO
T12EZ		K	V	K	V	L	G	D	V	I	E	V	TOAC	G	NO	NO

Table 2.1 shows the peptide sequences. The original sequence of K11V is the model for the spin-labelled peptides.[67] We prepared the three TOAC variants (T0EZ, T5EZ, T12EZ) with acceptable yields and high purity. The coupling of the bulky TOAC was done under special conditions: The amount of the precursor was adjusted, a different activator (1-Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU) was applied and a longer coupling time was allowed (see

SI); the variants we synthesized also differ from K11V by the Glycine residues at the C-terminal part of the sequence, introduced to facilitate the synthesis.

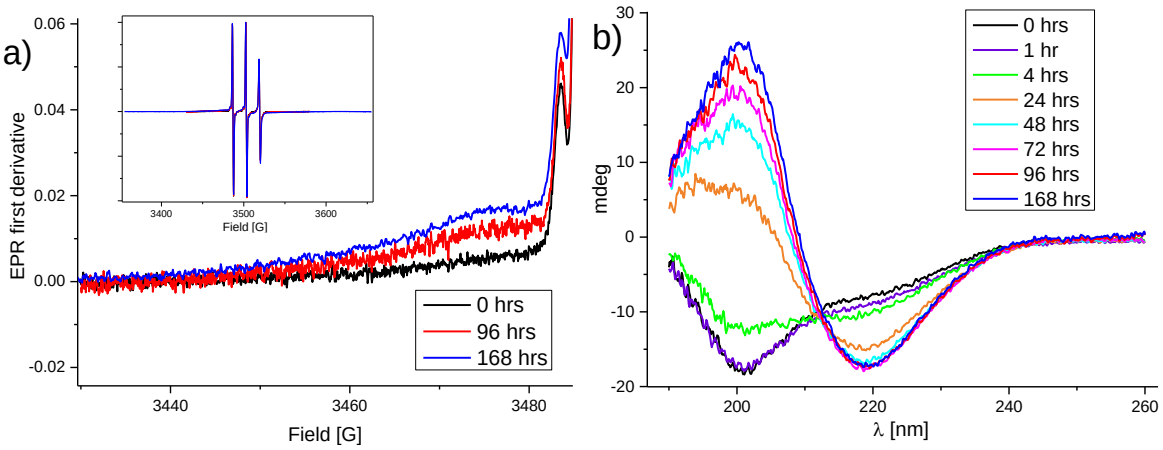

Figure 2.2 *(a) The 9 GHz spectra of T0EZ for three different aggregation times. The spectra are normalized to the maximum intensity (central line). The insert shows the full field-sweep spectra characterized by the usual three lines (derivative) for a nitroxide radical in solution; the main image shows the enlarged version of the low-field part of the spectra. At B_0 = 347.7 mT : broad peak, for details see text. (b) The CD spectra of T0EZ at different aggregation times.*

Figure 2.2 shows the cw-EPR and CD spectra of T0EZ for different aggregation times. Figure 2.2a focuses on the low-field region of the EPR spectra, where the changes are most obvious, the full spectra are shown as an insert. The intensity of the broad peak at 347.7 mT increases with the time of aggregation, while the intensity of the narrow lines decreases relative to that of the broad peak. The broad peak indicates the formation of T0EZ aggregates as described in the discussion.

Figure 2.2b shows the CD spectra taken during the aggregation of the same sample (see SI). At time zero the spectrum has a minimum at 201 nm, showing that the majority of the peptide is in a random-coil configuration. With increasing aggregation time, the minimum at 201 nm disappears and is replaced by a maximum at the same position. This newly developed maximum is characteristic for a β-sheet structure[94].

The same experiments were conducted for the other two TOAC peptides (T5EZ and T12EZ). The EPR spectra for both of them presented no visible broad peaks and were devoid of changes when the sample was subjected to the conditions under which the T0EZ spectra changed. The CD spectra at time zero showed two minima at 201 nm and 219 nm, similar to the time zero spectrum of T0EZ. The shape of the CD spectra did not change over time.

2.3 Discussion

We have investigated a set of spin-labelled peptides that resemble the K11V peptide. The purpose of our research was to check if the variants containing the TOAC spin label could be synthesized and if they still retained their aggregating behavior. All three variants were synthesized by Solid Phase Peptide Synthesis without problems. We used preloaded resins containing Glycine to facilitate the first couplings, which results in C-terminal Glycines in the sequence. The presence of TOAC didn't significantly reduce the yield of the reaction.

One of the peptides, T0EZ, showed aggregation, according to measurements by means of EPR and CD. Aggregates show up as a broad peak in the EPR spectra, reflecting the increased rotational correlation time of the oligomers as compared to the monomers. The intensity of the broad peak increases with time, showing that the amount of aggregates increases during the aggregation time. The changes in the CD spectra are also fully consistent with the aggregation: the transition of the random coil monomers to β-sheet oligomers, presumably similar to the oligomers shown in Figure 2.1c, is clearly seen in the development of the CD spectra.

As T5EZ and T12EZ showed none of the spectral changes observed for T0EZ we conclude that these peptides do not aggregate. To explain why T5EZ does not aggregate, we note that in the oligomer (Figure 2.1c) the TOAC would end up in the middle of the β-strands of the oligomer (see Figure 2.1c). Given that TOAC has a known propensity to induce α-helices or 3_{10} helices,[83,93] and the oligomer has a β-sheet structure, TOAC in that position may well interfere with oligomer formation. In addition, the TOAC residues of adjacent peptides could come close to each other in the oligomers, further inhibiting oligomer formation. The absence of aggregation in the T12EZ samples is more difficult to explain and needs to be investigated further.

We demonstrated that TOAC can be introduced at specific positions of the peptide sequence using SPPS, and that, for the oligomer formation intended here, the place must be chosen carefully not to alter the properties of the peptide. We show that EPR, combined with CD, is a powerful tool to study of the aggregation of such peptides.

2.4 Supporting information

2.4.1 Synthesis and Characterization of Peptides

All chemicals were commercial products of the best quality available and, unless otherwise indicated, they were used without any further purification. The K13G peptide was purchased from tebu-bio (Heerhugowaard, The Netherlands).

9-Fluorenylmethoxycarbonyl(Fmoc)-amino acids, Fmoc-Gly-Wang Tentagel resin and the other chemicals used for the solid phase peptide synthesis were purchased from Sigma Aldrich. 2,2,6,6-Tetramethylpiperidine-N-oxyl-4-(9-fluorenylmethyloxycarbonyl-amino)-4-carboxylic acid (Fmoc-TOAC-OH) and H-Gly-Wang resin were supplied by Iris Biotech (Germany).

The peptide sequences were assembled on AB433A Peptide Synthesizer, using (0.05 mmol) of Gly-Wang resin (substitution 0.5 mmol/g). For all amino acids except TOAC we use 5 equivalents (0.25 mmol) of each AA for the synthesis. Fmoc deprotection is done 4 times (3 minutes each) by adding (2.5 mL) of a solution of 20 % piperidine (PIP) in N-methylpyrrolidone (NMP). Couplings were performed using *2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate* (HCTU) as an activator and N,N-Diisopropylethylammine (DIPEA) as base. We used 1 equivalent of HCTU and 2 equivalents of DIPEA for 1 equivalent of AA. 1 mL of a solution (0.25 M) of HCTU, (0.5 mL) of a solution 1 M of DIPEA and (0.5 mL) of NMP are added to the resin for the coupling reactions. Each coupling reaction lasted 2 hrs.

The TOAC spin label was treated differently. Only 2 equivalents of AA were added for 1 equivalent of resin. Instead of HCTU, (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU) was used as activator and (400 µL) of its solution (0.5 M) were put directly inside the cartridge with the spin label together with (600 µL) of NMP. The coupling in this case lasted 4 hrs.

For the AA introduced immediately after the TOAC we used a double coupling, keeping the same conditions as for the rest of the sequence. The Fmoc absorption at 301 nm was followed to check the status of the synthesis after each coupling.

At the end of the synthesis the resin was dried by washing it with dichloromethane (DCM). To cleave the peptide from the resin (2 mL) of a solution 95% trifluoroacetic acid + 5% water was used. A small amount of peptide was cleaved from the resin and characterized by LC-MS.

Unless otherwise indicated, the peptides were purified by semi-preparative HPLC.

Analytical HPLC separation was carried out on a LCQ Advantag Thermo Finnigan LC-MS system with UV-Vis and Ion-trap mass detectors. The column used was a C-18 Gemini (4.6

x 50 mm, 3 µm particle size) from Phenomenex. The mobile phase A (H_2O) and B (acetonitrile, MeCN) and C (aqueous 1 % Trifluoroacetic acid, TFA) were used for preparing binary gradients. Elution condition: A 72 % B 18 % C 10 %, linear gradient B 18-33 % in 10 min. Flow rate 1 mL/min.

Crude peptide purifications were performed on a Gilson HPLC preparative system from Phenomenex with a semipreparative Gemini C_{18} column (10 x 250 mm) with UV-Vis detector. The mobile phase A (H_2O) and B (acetonitrile, MeCN) and C (aqueous 1 % Trifluoroacetic acid, TFA) were used for preparing binary gradients. Elution condition: A 82 % B 18 %, linear gradient B 18-33 % in 10 min, Flow rate 5 mL/min.

The lyophilization was done on a Christ Alpha 2-4 LO lyophilizer with a Christ RVC 2-25 rotor.

All TOAC peptides had a high level of purity.

T5EZ**:** yield 2.0%; LC-MS (C18) ^tR 4.56 min; purity > 95%; Mass: calculated for $C_{61}H_{108}N_{15}O_{18}$ $[M+H]^+$ 1339.61, found: 1339.47

T0EZ**:** yield 10.8%; LC-MS (C18) ^tR 4.68 min; purity > 95%; Mass: calculated for $C_{69}H_{122}N_{17}O_{20}$ $[M+H]^+$ 1453.794 , found: 1453.53

T12EZ**:** yield 1.4%; LC-MS (C18) ^tR 4.85 min; purity > 95%; Mass: calculated for $C_{69}H_{122}N_{17}O_{20}$ $[M+H]^+$ 1453.794 , found: 1453.60

2.4.2 Electron Paramagnetic Resonance

The 9 GHz, continuous-wave EPR spectra were recorded using an ELEXSYS E680 spectrometer (Bruker, Rheinstetten, Germany). The measurements were done under the following conditions: room temperature, a microwave power of 0.63 mW and a modulation amplitude of 0.15 mT at a modulation frequency of 100 kHz. The time expended on each measurement was adapted according to the spectral lineshape, i.e., the aggregation time. For the starting point of the aggregation 30 min were expended, and up to 5 h for samples at the end of the aggregation series. Glass micropipettes of a volume of 50 µL (Blaubrand Intramark, Wertheim, Germany) were filled with 20 µL of the sample for each measurement. The spin concentration was determined by comparing the double integral of the EPR spectra with the double integral of a reference sample (MTSL, 100 µM).

2.4.3 Circular Dichroism

Circular Dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the differences in the absorption of right-handed versus left-handed circularly polarized light that arise due to the chirality of a molecule. The two types of circularly polarized light are absorbed to different extents. In a CD experiment, the same intensity of left and right circularly polarized light at a certain wavelength are alternately radiated into a sample. In the presence of chirality, one of the two polarized beams is absorbed more strongly than the other. The difference in absorption depends on the wavelength and it is measured as the CD spectrum. It has been shown that CD spectra between 260 and approximately 180 nm can be analyzed for the different secondary structural elements of proteins: alpha helix, parallel and antiparallel beta sheet, turn, and other. The ability of CD to give a representative structural signature makes it a powerful tool in modern biochemistry.[95]

The CD spectra were obtained using a J-815 CD Spectrometer (Jasco Benelux, Utrecht, The Netherlands). The measurements were carried out at room temperature under the conditions of 260 nm - 190 nm wavelength range, continuous scanning mode, a band width and a data pitch of 1 nm each, a speed of 20 nm/min and a total of five accumulations for each measurement. A 2-mm-path-length cuvette was used for measurements. A sample of 40 μL of the aggregation solution was taken and diluted ten times in water in order not to saturate the photodetector of the spectrometer.

2.4.4 Protocol of Aggregation Experiments

Samples were prepared as follows: The powder of the TOAC EZ peptides was dissolved in Milli-Q water, in order to get a nominal peptide concentration of 500 μM. The experiments were carried out during a week. After an initial measurement taken at the time the spin-labelled-peptide powder was diluted in water (called time-zero measurement), samples with a total volume of 560 μL in 1.5 mL Eppendorf tubes were aggregated on a thermomixer (Thermomixer comfort) with a speed of 1000 rpm at 293 K. At each the time point, a 20 μL sample was drawn for an EPR measurement, one of 40 μL for CD and 10 μL were frozen for future experiments. The time points were: one hour, four hours, one day (24 h), two days (48 h), three days (72 h), four days (96 h) and seven days (168 h).