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

Zurlo, E.

6 Synthesis and characterization by EPR of backbone-labeled Amyloid β peptides and influence of an antiamyloidogenic cyclic D,L-α-peptide as potential inhibitor for their aggregation

The Amyloid β peptide (Aβ) is involved in Alzheimer's disease, the most common neurodegenerative disease. This chapter reports the synthesis of two TOAC, 2,2,6,6-tetramethyl-N-oxyl-4-amino-4-carboxylic acid, labeled Aβ peptides, T0Aβ with the spin label at the N-terminus, and T26Aβ with the spin label at position 26 in the sequence. The TOAC spin label incorporates directly into the backbone of peptides, an advantage to determine the aggregation of the peptide. To monitor the aggregation, the rotational correlation time (τ_r) is measured by 9 and 95 GHz EPR. Both constructs aggregate, however, T26Aβ significantly less than T0Aβ. We show that the antiamyloidogenic cyclic D,L-α-peptide CP-2[188,189] interacts with T0Aβ and T26Aβ, possibly as a multimer. Above a ratio of 5 : 1 of CP2 to Aβ, CP2 blocks the formation of fibrils.

6.1 Introduction

Alzheimer's disease is a neurodegenerative disease affecting an increasing amount of the aging population[190]. There is currently no cure, making the search for mechanisms of the disease urgent and timely. The aggregation of the amyloid β peptide, that derives from the misprocessing of the amyloid precursor protein (APP) is one of the central features of the disease[191–196]. As also the physical-chemical mechanisms of amyloid aggregation are poorly understood, research techniques to investigate the physical-chemical mechanisms of amyloid aggregation are necessary.

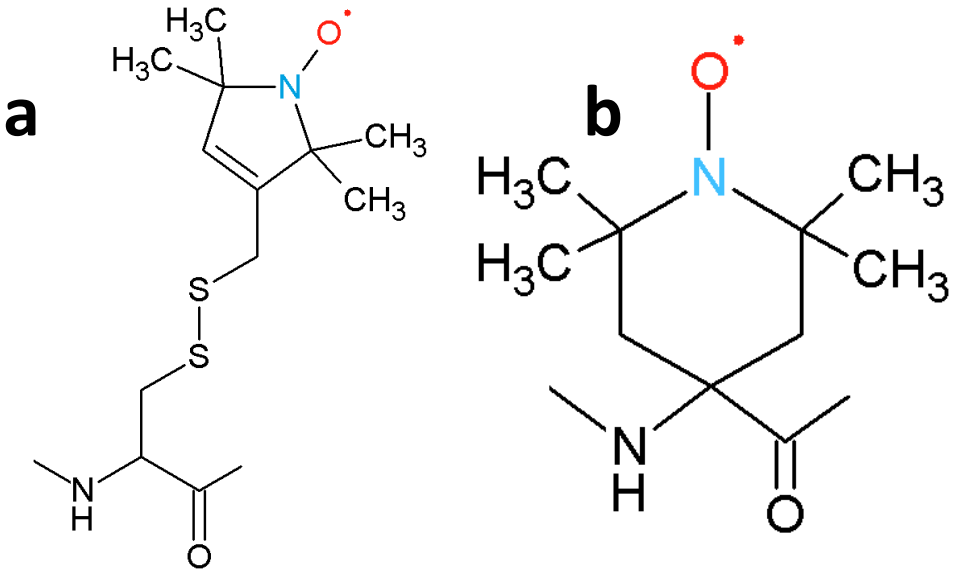

Figure 6.1 *a) Molecular structure of the MTSL spin label attached to a cysteine side-chain. b) Molecular structure of the TOAC spin label.*

Here we want to make use of Electron Paramagnetic Resonance (EPR) to study amyloid aggregation. Specifically, we will employ the sensitivity of continuous wave EPR to the rotational mobility of an object, which is related to its size and thereby can measure the size-increase of the aggregating peptides[45–47,57,197]. The rotational correlation time can be measured, if the object contains a nitroxide spin label. In the context of the present study this means that the amyloid peptide has to be labeled with a nitroxide spin label, see Figure 6.1.

Usually spin labels, such as the *S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL) are used. Such labels are coupled to a cysteine in the protein, a cysteine that is introduced by site-directed spin labeling[45,57]. The disadvantage of this approach is the local mobility of such spin labels, caused by the rotational freedom about the single bonds connecting the nitroxide to the cysteine residue, and thereby the backbone of the protein, see Figure 6.1a. As EPR probes the mobility of the N-O group of the nitroxide, this decoupling, local mobility, limits the access

to mobility information on the particle, i.e. the aggregates that the peptide forms. We therefore resorted to the 2,2,6,6-tetra-methylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) spin label. In TOAC, the nitroxide is directly coupled to the peptide backbone, see Figure 6.1b, thus avoiding the problem of local mobility.

To investigate the aggregation of amyloid peptides using this approach, we synthesized the peptides shown in Table 6.1 by SPPS (Solid Phase Peptide Synthesis). The peptides are based on the 40-amino acid long APP fragment Aβ-40. They contain the TOAC spin label in two strategically chosen positions: At the N-terminus (T0Aβ) and at position 26 (T26Aβ), replacing the Asn in the AβWT sequence. These constructs enable us to monitor the end (T0Aβ) and the central part (T26Aβ) of the peptide.

We investigate these peptides by continuous wave EPR in liquid solution. To maximize the sensitivity and resolution, standard 9 GHz EPR is combined with high-field, 95 GHz EPR. The lineshape of the EPR spectra obtained is sensitive to the nitroxide motion in the time regime of 0.1 ns to 10 ns and thereby is relevant to the aggregation process as described below.

In the present study we investigate if and how TOAC influences the aggregation of Aβ, as a first characterization of the synthesized constructs. As a first application, we study the interaction of T0Aβ and T26Aβ with a cyclic D,L-α-peptide (CP-2) (Table 6.1), a recently discovered inhibitor of Aβ aggregation, considered as a possible drug candidate[188,189].

We find that T0Aβ aggregates significantly faster and with higher yields than T26Aβ. We show that the interaction of Aβ with CP-2 can be detected and that multiple CP-2 peptides must be involved in the interaction.

Table 6.1 *Sequences of peptides. The spin label TOAC (2,2,6,6-tetra-methylpiperidine-1-oxyl-4-amino-4-carboxylic acid) is abbreviated as T.*

	Sequence[a]
Aβ-40 WT	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
T0Aβ-40	T-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
T26Aβ-40	DAEFRHDSGYEVHHQKLVFFAEDVGS-T-KGAIIGLMVGGVV
CP-2	[l-J-w-H-s-K]

[a] Capital and lower case letters represent L- and D-amino acid residues, respectively. Square brackets indicate a cyclic structure.

6.2 Materials and methods

6.2.1 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 Abeta40 WT and Abeta42 WT peptide were purchased from tebu-bio (Heerhugowaard, The Netherlands).

9-Fluorenylmethoxycarbonyl(Fmoc)-amino acids, Fmoc-Val-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-Val-Wang resin were supplied by Iris Biotech (Marktredwitz, Germany).

The peptide sequences were assembled on AB433A Peptide Synthesizer (Applied Biosystems, Foster City, CA, USA), using 0.05 mmol of Gly-Wang resin (substitution 0.24 mmol/g). For all amino acids except TOAC we use 10 equivalents (0.5 mmol) of each AA for the synthesis. Deprotection by Fmoc was done 3 times (2 minutes each) by adding 2.5 mL of a solution of 20 % piperidine (PIP) in N-methylpyrrolidone (NMP). Couplings were performed using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) as an activator and N-Methylmorpholine (NMM) as base. We used 1 equivalent of HATU and 2 equivalents of NMM for 1 equivalent of AA. 2 mL of a solution of HATU (0.225 M in NMP), 0.5 mL of a solution of NMM (1 M in NMP) and 1 mL of NMP:DMSO (1:1) were 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. HATU was used as activator and 400 μL of its solution (0.5 M in NMP) 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 step.

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 (TFA) + 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 (Torrance, California). The mobile phase A (H_2O), B (acetonitrile, MeCN) and C (aqueous 1 % TFA) was used for preparing ternary

gradients. Elution condition: A 80 % B 10 % C 10-90 %, linear gradient B 18-33 % in 10 min. Flow rate 1 mL/min.

Crude peptide purifications were performed on a Gilson HPLC preparative system with a semipreparative Gemini C_{18} column (10 x 250 mm) from Phenomenex with UV-Vis detector. The mobile phase A (aqueous 1 % trifluoroacetic acid, TFA) and B (acetonitrile, MeCN) 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 (Salm&Kipp, Breukelen, Netherlands) with a Christ RVC 2-25 rotor. All TOAC peptides had a high level of purity as shown by single band elution and mass spectrometry.

T0AB40: yield 11.996%; LC-MS (C18) ^{t}R 7.06 min; purity > 95%; Mass: calculated for $C_{61}H_{108}N_{15}O_{18}$ $[M+H]^+$ 4529.14, found: 4529.40

T26AB40: yield 3.152%; LC-MS (C18) ^{t}R 7.27 min; purity > 95%; Mass: calculated for $C_{69}H_{122}N_{17}O_{20}$ $[M+H]^+$ 4440.06 , found: 4440.00

6.2.2 Protocol for the aggregation experiments

Samples were prepared as follows: The powder of the lyophilized spin-labelled Aβ peptides was treated with a solution of 30 % NH_3 in H_2O for 3 hours at RT to restore the nitroxide radical. The solution was then lyophilized once again. The resulting powder was dissolved in DMSO to obtain stock solutions of concentration 3 mM. Of these stock solutions, 33 µL were pipetted into 1.5 mL Eppendorf tubes. To ensure the monomeric state of the peptide, we added 100 µL of NaOH 10 mM to the Eppendorf tubes[198]. After 2 minutes of sonication in an ice bath, chilled 100 µL of PBS 100 mM buffer containing 25 µM ThioT was added. To neutralize the base we added 100 µL of HCl 10 mM to obtain a final pH of 7.4. At this point, milliQ water and DMSO were added to obtain a final volume of 500 µL. The final concentrations are Aβ 200 µM, PBS buffer 20 mM, ThioT 5 µM with a final concentration of DMSO of 10 %. Samples containing the inhibitor CP-2 were prepared following the same protocol. The stock solution of CP-2 was prepared by dissolving the lyophilized powder in DMSO to a final concentration of 39 mM. Of this stock solution, the appropriate amounts of CP-2 were added to the Aβ solution to obtain the desired ratio of peptide and inhibitor. If required, DMSO was added to obtain a final 10 % DMSO concentration in all samples. Aggregation experiments were carried out during one week, unless noted otherwise.

6.2.3 ThioflavinT fluorescence plate reader experiments

The samples were monitored by the standard Thioflavin T (ThioT) fluorescence assay[107]. The Eppendorfs were put for 2 minutes of sonication in ice bath, then samples were pipetted into a 96 wells plate, holding 200 µL each. Each sample was measured in duplicates. Fluorescence measurements were recorded with an CLARIOStar Plus (BMG LABTECH GmbH, Allmendgrün 8, 77799 Ortenberg, Germany) microplate reader every 10 min for a week, using excitation and emission wavelengths of 444 ± 10 and 485 ± 10 nm, respectively. The plate was held at +37 °C, and the wells were automatically shaken 30 seconds before each measurement step. The fluorescence increase was measured with respect to the ThioT blank without the peptide.

6.2.4 EPR measurement conditions

The remaining sample solutions in the Eppendorf tubes were kept on ice to be measured by EPR. After an initial measurement taken at the time the sample was prepared ($t = 0$), the remaining samples of 80 µL in 1.5 mL Eppendorf tubes were aggregated on a thermomixer (Eppendorf, Thermomixer comfort, Waltham, MA USA) at 310 K. The EPR of the aggregated sample was measured after one week to observe the aggregation.

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 (293 K), a microwave power of 0.63 mW and a modulation amplitude of 0.2 mT at a modulation frequency of 100 kHz. The time expended on each measurement was adapted according to the spectral lineshape. 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). The spin concentrations determined from the double integrals were 60 µM for T26Aβ and 80 µM for T0Aβ to an expected 200 µM , giving an activity of ≈ 30 % and ≈ 40 % respectively.

The 95 GHz EPR spectra were recorded at room temperature on a Bruker ELEXSYS E680 spectrometer using a home-built probehead with a single-mode cavity specially designed for cw measurements. Acquisition parameters: microwave frequency 94.04 GHz, microwave power 0.63 µW, modulation amplitude 1 mT, modulation frequency 6 kHz. Total measurement time: approximately 5 hours.

6.2.5 Simulations of EPR spectra

MATLAB (version 9.4.0.813654, R2018a, The MathWorks, Inc., Natick, MA, USA) and the EasySpin package (5.2.25) were used for simulations of the EPR spectra.[48] The parameters of the simulation were manually adjusted to agree best with the experimental spectra. For all simulations, an isotropic rotation of the nitroxide ($S = 1/2$) was utilized. The following g-tensor values were used: $g = [2.0086\ 2.0059\ 2.0020]$. These values were obtained from M. Hashemi Shabestari et al.[108], and we used these values for all other simulations. The spectra were simulated with a superposition of three components: a fast and medium fractions using the "Chili" function and a slow fraction using the "Pepper" function. For T0Aβ the principal values of the ^{14}N hyperfine coupling tensor were $A_{xx} = A_{yy} = 13$ MHz and $A_{zz} = 112.7$ MHz for the fast component, $A_{xx} = A_{yy} = 13$ MHz and $A_{zz} = 107.5$ MHz for the medium component, and $A_{xx} = A_{yy} = 13$ MHz and $A_{zz} = 95$ MHz for the slow component.[108] For T26Aβ the values were $A_{xx} = A_{yy} = 13$ MHz and $A_{zz} = 110.7$ MHz for the fast component, and $A_{xx} = A_{yy} = 13$ MHz and $A_{zz} = 95$ MHz for the slow component. For the 9 GHz spectra, a Gaussian component with a linewidth of 0.1 mT was used for the fast component and medium components, and 0.65 mT for the slow component. For the 95 GHz spectra of T26Aβ, a Gaussian line with a width of 0.12 mT was used for the fast component and of 0.15 mT for the slow component was applied. The τ_r of the fast component was chosen by simulating the narrow lines of the time 0 samples without CP2. Optimal τ_r values of the medium and slow components were derived from later time-point spectra and then kept constant for all simulations, with and without CP-2.

6.3 Results

The two Aβ variants, T0Aβ and T26Aβ (sequences given in Table 6.1) were obtained in good yield and high purity (see Material and Methods). Reactivation of the nitroxide radical resulted in the 40 ± 5 % active nitroxide for T0Aβ and 30 ± 15 % for T26β. Here we report the first characterization of the aggregation behavior for both peptide variants.

To detect fibrilization, ThioT fluorescence was used. For T0Aβ ThioT fluorescence shows a plateau phase after 24 hours of aggregation, showing the formation of fibrils[199]. For T26Aβ the ThioT signal increases as well, but the fluorescence of the plateau is approximately 10 % of that of T0Aβ at comparable concentrations (see Figure S6.3). The ThioT results show that after a few days, aggregation is complete. Therefore, the EPR experiments, described in the following, were performed immediately after dissolving the peptide in buffer, referred to as "before aggregation" and after 7 days of aggregation referred to as "aggregated sample".

Figure 6.2 shows the 9 GHz EPR spectra of T0Aβ and T26Aβ before and after aggregation. The spectra have the three-line pattern typical of nitroxide spin labels. The spectra (black) of T0Aβ and T26Aβ before aggregation differ from each other. The lines of T26Aβ

are broader than those of T0Aβ, which shows a lower mobility at the center of the peptide compared to the N-terminus.

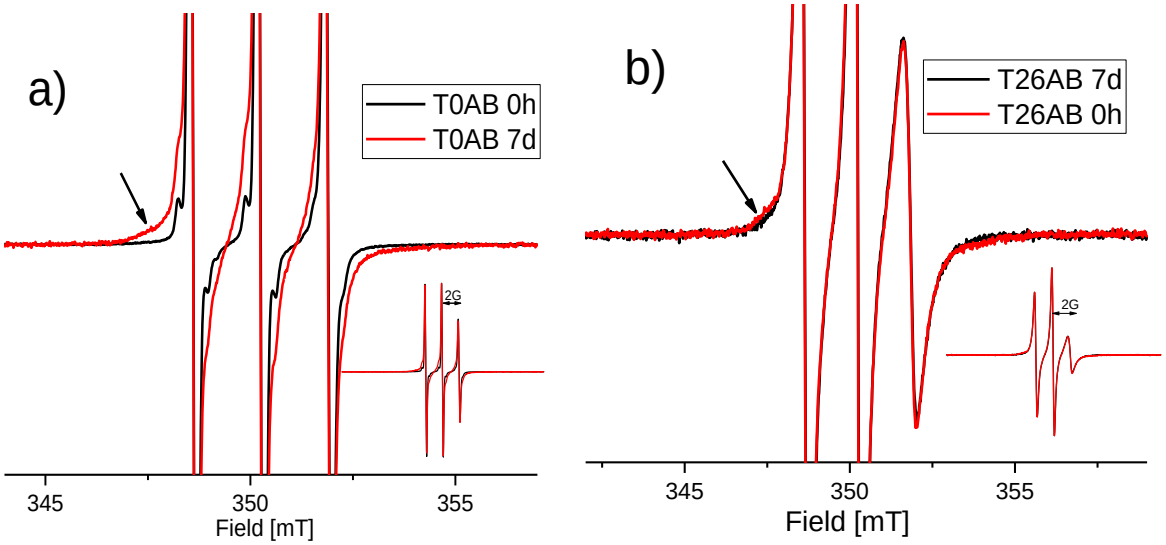

Figure 6.2 *Comparison of EPR spectra of TOAC Aβ constructs before and after aggregation. Full spectra: inset. Zoomed-in spectra: Amplitude expanded four-fold. a) Spectra of T0Aβ at start of aggregation (black) and after 7 days (red). b) Spectra of T26Aβ at start of aggregation (black) and after 7 days (red).*

After aggregation (conditions see Material and Methods), the T0Aβ spectrum (Figure 6.2a, red) shows of an additional broad component, marked by the arrow, representing a component with lower mobility. The T26Aβ spectrum after aggregation (Figure 6.2b, red) closely resembles the one at time 0, with much smaller deviations from the initial spectrum than observed for T0Aβ.

By spectral simulation more details are obtained: All spectra can be simulated with maximally three components, a fast, a medium and a slow component. Figure 6.3 shows the shape of the components and their agreement with the experimental spectra. In Figure 6.3a and b the components by 9 GHz EPR are shown. For T26Aβ we confirmed these components by 95 GHz EPR (Figure 6.3c).

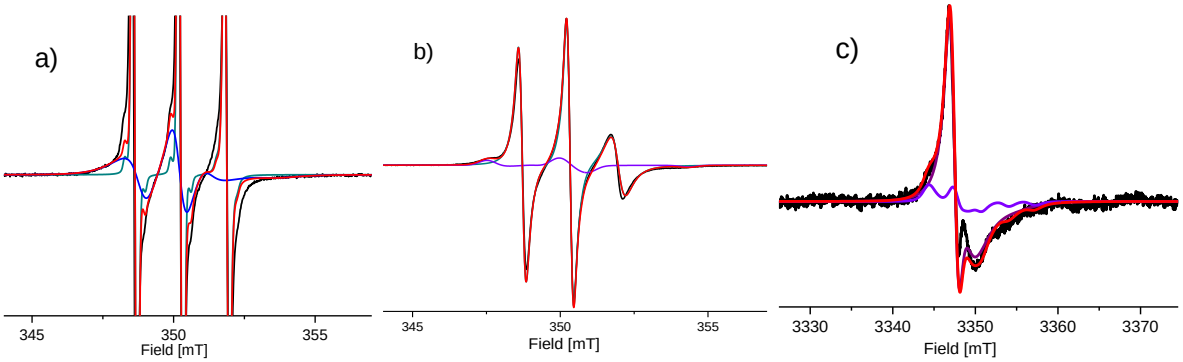

Figure 6.3 *Spectral components used in the simulation of the 9 GHz EPR spectrum of T0Aβ (amplitude zoomed-in four-fold, 7 days aggregation) and T26Aβ (10 days aggregation), and 95 GHz for T26Aβ (10 days aggregation). Experimental spectra (black), fast component (dark cyan), medium component (blue), slow component (violet). Total simulation (red). For further details see the text.*

In Table 6.2 the τ_r values of these components are given and compared to the τ_r value of R1Aβ from M. Hashemi Shabestari et al.[65] The Aβ40 variant R1Aβ contains an MTSL attached to a cysteine at the same position as the TOAC in T0Aβ. The fastest τ_r , assigned to monomeric Aβ, is roughly seven times faster for T0Aβ than for T26Aβ. The fast component for T0Aβ has a smaller τ_r than R1Aβ, even though we would have expected a higher mobility for the MTSL spin label.

Table 6.2 *The rotational correlation times of the components of T0Aβ, T26Aβ and their percentages before and after aggregation. Comparison with the τ_r of MTSL labeled Aβ from M. Hashemi Shabestari et al.* [65]

	T0Aβ			**T26Aβ**			**R1Aβ**[65]
	[ns]	time 0 [%]	7 days [%]	[ns]	time 0 [%]	7 days [%]	[ns]
τ_r fast	0.151 ± 0.004	82 ± 6	36 ± 2	1.071 ± 0.050	100%	93 ± 1	0.19 ± 0.02
τ_r medium	3.162 ± 0.410	18 ± 6	64 ± 2	-	-	-	
τ_r slow	-	-	-	> 10	-	7 ± 1	

In T0Aβ a slower component of τ_r (medium) is already present at the start of the aggregation with a contribution of ca. 20 %. Considering that we assigned the fastest τ_r to the monomeric Aβ, this medium component could be related to the presence of some aggregates. After aggregation, the amount by which this fraction contributes to the spectra has increased to 64 %, which suggests that more than half of the T0Aβ has aggregated.

In T26Aβ at the start of aggregation only one component is present, again assigned to monomers. After aggregation this fast component is still dominant, with the appearance of a small percentage of a component with lower mobility, which we refer to as the slow component.

In Figure 6.4 the effect of CP-2 on T0Aβ and T26Aβ is shown. Spectra with the inhibitor (CP-2 5:1 molar ratio) show additional broad lines at the low and high field side of the spectra, e.g. at $B_0 = 347.5$ mT and 354 mT in Figure 6.4b and d. This shows that the mobility of the spin labels is reduced by the interaction with CP-2.

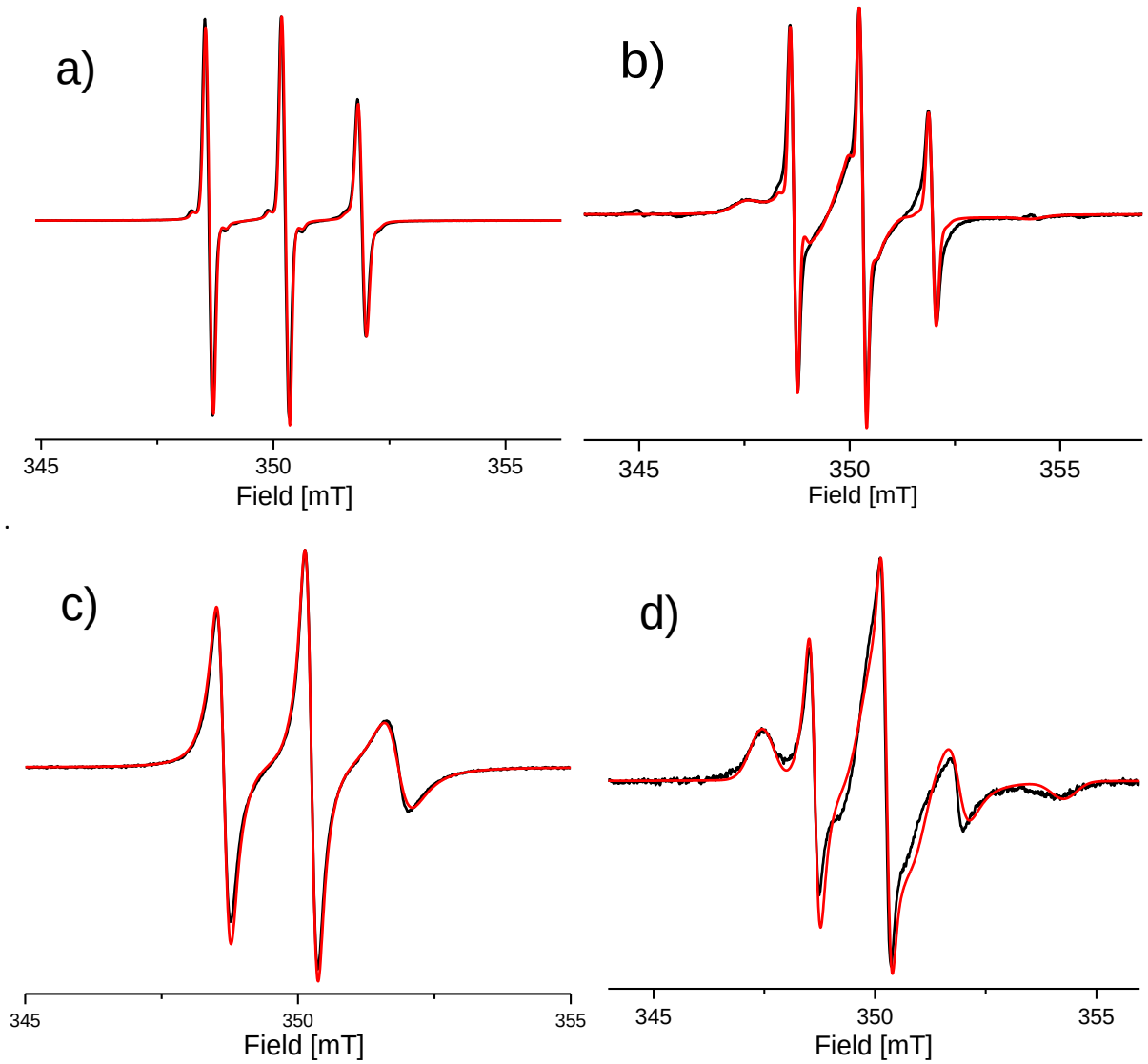

Figure 6.4 *Comparison of EPR spectra of TOAC Aβ constructs with and without CP-2 before aggregation. a) T0Aβ 200 μM. b) T0Aβ + 1 mM CP-2. c) T26Aβ 200 μM. b) T26Aβ + 1 mM CP-2. Black: experimental spectra. Red: simulations.*

In the Table 6.3 the τ_r values of these components are given. The fast and medium components are identical to those given in Table 6.2. The slow component is now present also for T0Aβ. Figure 6.5 shows the shape of these components for the spectra shown in Figure 6.4b and d. Compared to the spectra of the T0Aβ without CP-2, the interaction with the inhibitor results in a new component (Figure 6.5a, blue line) with a larger τ_r compared to the fast and medium components. Figure 6.5b shows the components used to simulate the T26Aβ peptide in the presence of CP-2.

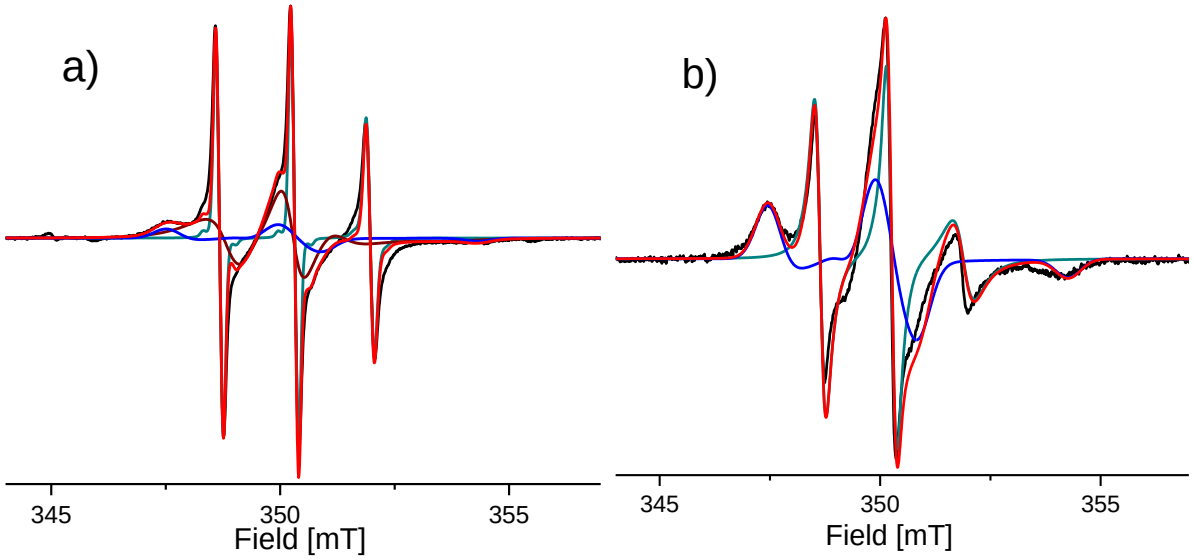

Figure 6.5 *Spectral components used in the simulation of the 9 GHz EPR spectrum of T0Aβ 200 μM + 1 mM CP-2. Experimental spectra (black), fast component (dark cyan), medium component (wine), slow component (blue). Total simulation (red). For further details see the text.*

Table 6.3 *Rotational correlation times of the components of T0Aβ and T26Aβ in the presence of CP-2 and comparison with the τ_r of MTSL labeled CP-2 (R1CP-2).*

	T0Aβ	**T26Aβ**	**R1CP-2**
	[ns]	[ns]	[ns]
τ_r fast	0.151 ± 0.004	1.071 ± 0.050	0.141 ± 0.003
τ_r medium	3.162 ± 0.410	-	
τ_r slow	> 10	> 10	

The amounts by which the individual components contribute to the spectra are plotted in Figure 6.6. In addition to the molar ratio of Aβ : CP-2 of 1 : 5, two more ratios are tested, leading to the full set of 1:0.5, 1:1 and 1:5 (for individual spectra, see Figures S6.1).

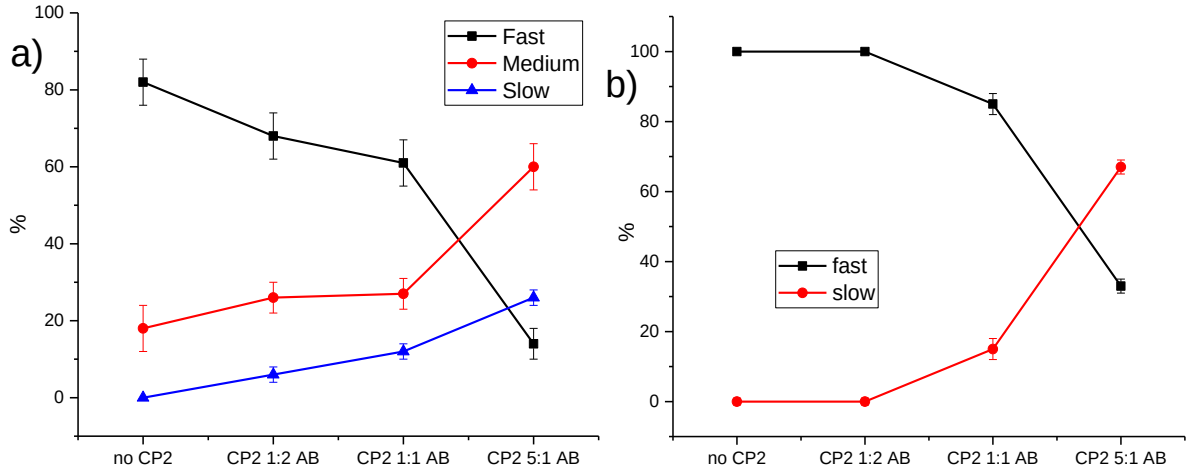

Figure 6.6 *Effects of various ratios of CP-2:Aβ on EPR spectra. a) Amount of fast, medium and slow components in the EPR spectra simulations of T0Aβ: fast (black), medium (red) and slow (blue). b) Amount of fast and slow components in the EPR spectra of T26β: fast (black), slow (red). The lines are a guide to the eye. For details see text.*

The difference between the contribution of spectral components in the absence and presence of CP-2 on T0Aβ and T26Aβ at a CP-2 :Aβ ratio of 1:2 are considered not significant, given the experimental errors.

6.4 Discussion

We synthesized two variants of the Aβ peptide with the TOAC spin label in different positions in the Aβ chain: at the N-terminus (T0Aβ) and in a central position (T26Aβ) (Table 6.1). We use TOAC because it reports more faithfully on the backbone mobility of the peptide, however, it is also known to induce α-helical structures[77–79,82,93,200] and therefore may influence the peptide conformation and reactions. To test this we test the ability of the newly synthesized peptides to aggregate.

6.4.1 EPR properties of monomers

The nitroxide in T26Aβ has a higher τ_r value than that of T0Aβ. As both peptides should have almost the same molecular weight (only 2 % difference) and thereby the same volume, the Stokes-Einstein relation (Eqn. S6.1) predicts that both peptides should have similar τ_r values. The faster τ_r of T0Aβ therefore must reflect a higher local backbone flexibility at the N-terminus that in the middle of peptide.

This local mobility apparently is so high that the τ_r of the nitroxide in T0Aβ is smaller

than the one of R1Aβ[65,201]. That is surprising because for the MTSL spin-label the rotation about the single bonds linking the nitroxide to the peptide backbone (see Figure 6.1a) should dominate the motion, which is not possible for TOAC. It is possible that sample conditions, such as the high peptide concentration of 0.55 mM[65] contribute to this difference. An indication for such an effect is that the τ_r values differences of τ_r found for R1Aβ in another study[201] also differ from those in ref.[65]. Further experiments are needed to clarify this point. Also the center of the peptide is not devoid of local mobility: the τ_r expected from the molecular volume is 25 % longer than that measured for T26Aβ.

6.4.2 Influence of TOAC on aggregation behavior

Qualitatively, T0Aβ aggregates similarly to Aβ wild type. More detailed studies are needed to reveal in how far the time scale and the concentration dependence also agree. In contrast to T0Aβ, T26Aβ hardly shows signs of amyloid aggregation: ThioT levels remain low and immobilization, observed by EPR, occurs on a much lower level than for T0Aβ. As the literature does not report such a trend for the R1 derivatives of Aβ[65,201], we assume that the main factor is the presence of the TOAC amino acid. Further studies are needed to determine the role played by TOAC in such a context, however we can already indicate two possible factors combining to inhibit the aggregation: Crowding in the middle of the β-sheet structure of the fibril, and the α-helix-inducing properties of TOAC[77–79,82,93,200].

6.4.3 Cyclic peptide CP-2 as amyloid aggregation inhibitor

Having established that TOAC enables tracking the aggregation properties of Aβ at different positions, we use it to analyze the interaction between the labeled peptides and the CP-2 inhibitor. ThioT fluorescence (Figure S6.3) shows that the effect of CP-2 on the formation of fibrils for our TOAC labeled peptides is similar to what has been previously observed by Rahimipour et al. [188,189] for the Aβ WT: CP-2 inhibits Aβ aggregation. The EPR experiments confirm a direct interaction between CP-2 and the TOAC labeled Aβ peptides: A new component appears in both T0Aβ and T26Aβ spectra. This component has a τ_r that is larger than the τ_r in the absence of CP-2, which suggests a complete immobilization of the peptide, or at least a τ_r larger than 10 ns. In chapter 3 we have shown that by cw EPR the number of peptide units present in the aggregates can be estimated[197]. Following the same principle we tried to determine the composition of the aggregates and the limits of the detection. Considering the molecular weight of the TOAC labeled Aβ peptides (4500 Da, see Materials and methods), seven is the highest amount of units in an aggregate that would have a non-immobilized EPR spectrum in the absence of local mobility of the spin label. As CP-2 has a molecular weight (787 Da) that is lower

than that of Aβ, a maximum of 34 CP-2 units added to one Aβ peptide would result in immobilized spectra. The presence of CP-2 gives rise to a completely immobilized τ_r, so we can assume that T0Aβ and T26Aβ get partially trapped in large units formed with the inhibitor.

The results of the EPR experiments on the spin-labeled version of CP-2 (R1CP-2) (see SI) reveal that CP-2 indeed strongly interacts with itself. A dominant fraction of R1CP-2 has a spectrum (Figure S6.4) that is characteristic of strong spin-spin exchange, which suggests the interaction of more than ten R1 residues, with R1-R1 distances around 4-5 Å[202,203]. Such an interaction is fully consistent with the model of in-register-β-sheet stacking proposed by Rahimipour et al. [188,189]

6.5 Conclusions

Here we show that Aβ peptides labeled with TOAC at strategic positions open new ways to study the amyloid aggregation: The real-time measurement of rotational motion reveals different mobility at the two sites investigated in Aβ, giving a better understanding of the interactions involved in the aggregation process. The TOAC at the N-terminal position in Aβ (T0Aβ) seems to have no impact into the formation of fibrils, whereas the TOAC at center of the peptide partially inhibits its aggregation. The T26Aβ gives us a better understanding of the total mobility of the peptide, however it seems to influence the aggregation process.

We use these two labeled Aβ peptides to better understand the effect of the antiamyloidogenic cyclic D,L-α-peptide CP-2 on the aggregation process. We prove that CP-2, above a certain ratio with Aβ, forms instantaneously a construct that immobilizes the amyloid peptide and blocks the formation of fibrils. By using cw EPR on a MTSL labeled CP-2 (R1CP-2) we show that, under our experimental conditions, the inhibitor forms a macromolecular structure by itself, which suggests that, if present, Aβ gets trapped into such structures, blocking the fibril formation.

New strategic positions for the labels in Aβ could be used to better define their influence in the aggregation process. Such research could be used to better observe the presence of relevant Aβ forms, e.g. oligomers. Further experiments should be done to determine the structure of the Aβ-CP-2 aggregates and the exact source of the interaction of CP-2 with the Aβ peptide.

6.6 **Supporting information**

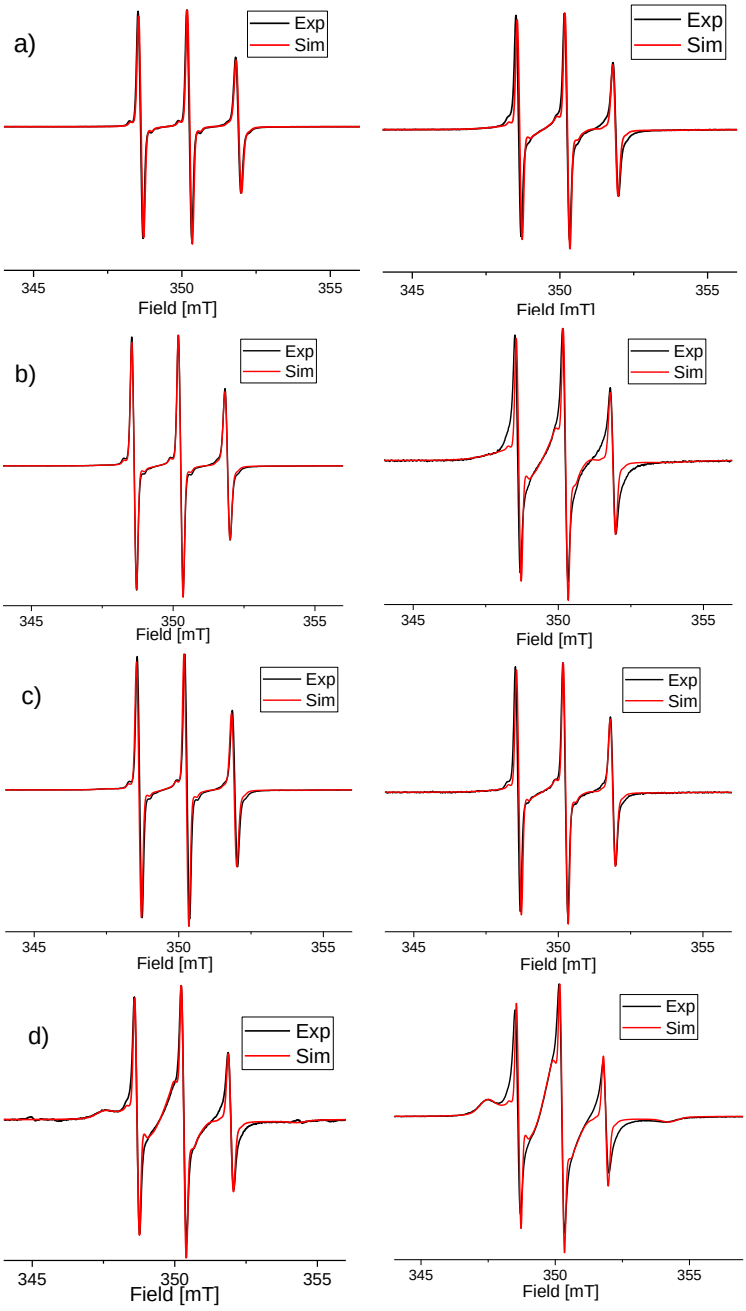

Figure S6.1 *Room temperature 9 GHz EPR spectra of T0Aβ 200 μM + CP-2 before and after aggregation. Left column: EPR spectra at time 0. Right column: EPR spectra after 7 days of aggregation. Spectra a) no CP-2, b) 100 μM CP-2, c) 200 μM CP-2, d) 1 mM CP-2. Black: Experimental spectra. Red: Simulated spectra. Remaining experimental conditions and detailed description: See main text.*

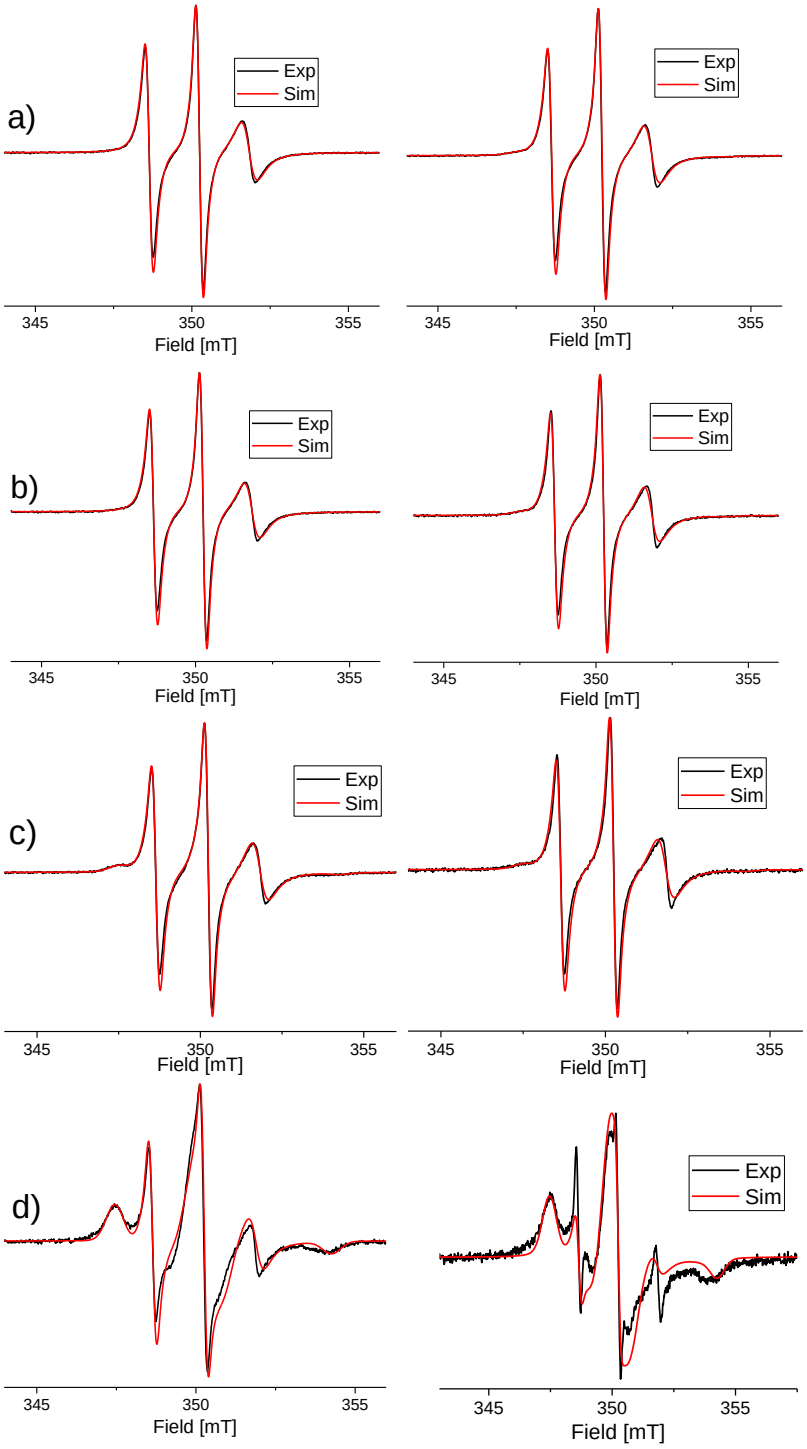

Figure S6.2 *Room temperature 9 GHz EPR spectra of T26Aβ 200 μM + CP-2 before and after aggregation. Left column: EPR spectra at time 0. Right column: EPR spectra after 7 days of aggregation. Spectra a) no CP-2, b) 100 μM CP-2, c) 200 μM CP-2, d) 1 mM CP-2.*

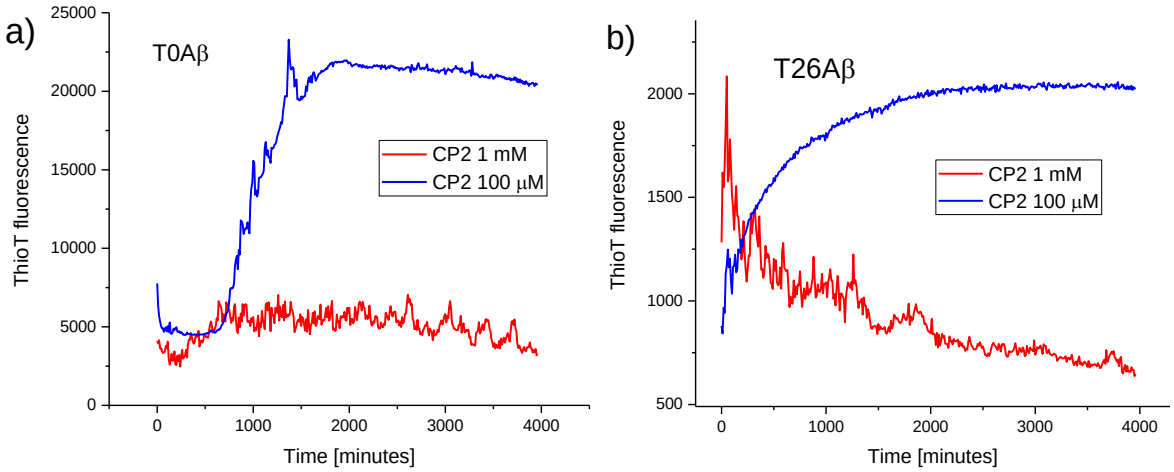

Figure S6.3 *Inhibitory effect of CP-2 on T0Aβ and T26Aβ detected by ThioT fluorescence in plate reader. a) ThioT fluorescence kinetics of T0Aβ 200 μM incubated for 3 days at 37 ºC in the presence of CP-2 at concentration 100 μM (blue) and 1 mM (red). b) ThioT fluorescence kinetics of T26Aβ 200 μM incubated for 3 days at 37 ºC in the presence of CP-2 at concentration 100 μM (blue) and 1 mM (red). For more details, see main text.*

6.6.1 Interpretation of τ_r values and molecular volumes

We used the Stokes-Einstein equation to interpret the τ_r values. This implies a spherical approximation for the particles:

$$\tau_r = \frac{4\pi\eta\alpha^3}{3kT} = \frac{\eta}{kT}V_{EPR} \qquad (S6.1)$$

In Eqn. (S6.1) k is the Boltzmann constant, T is the temperature (293 K), $\boldsymbol{\eta}$ is the viscosity of the solvent (1.05 cP for 10 % DMSO in water[204]) and α is the hydrodynamic radius.

The volumes of the peptide and its aggregates can be derived by using the molecular weight (MW) and a certain density of the proteins using Eqn. (S6.2):

$$V = \frac{MW}{N_A\rho} \qquad (S6.2)$$

Here, ρ is the protein density, N_A is Avogadro's constant.

By comparing the volumes obtained by Eqn. S6.1 with the ones relative to a certain molecular weight we can estimate the number of units comprising an aggregate. For more details, see Chapter 3 of this thesis.

6.6.2 EPR experiments on spin-labeled CP-2

To detect the influence of CP-2 on the aggregation of Aβ, initial experiments of spin labeled CP-2 (R1CP-2) with and without Aβ were performed. The nitroxide was attached

to the CP-2 to a cysteine in position 6 in the sequence via a iodoacetamide labeling reaction. Sequence R1CP-2: [l-J-w-H-s-R1]

The same protocol used to prepare the TOAC labeled peptide samples was used for the R1CP-2 samples, but the final concentrations of Aβ WT was 400 μM and R1CP-2 was 200 μM. As a reference, a sample of pure R1CP-2 at a concentration of 200 μM without Aβ was prepared.

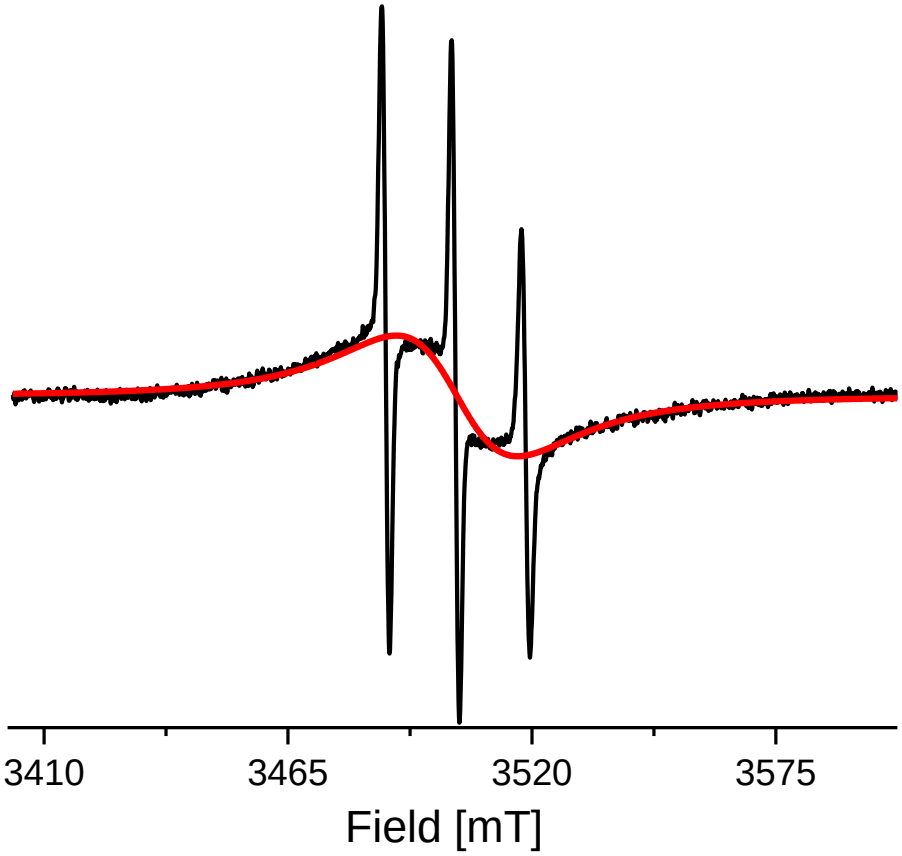

Figure S6.4 *Spin-spin exchange interaction in a aggregated solution of R1CP-2. Experimental 9 GHz EPR spectrum (black) and fitted Lorentzian derivative of the broadened signal (red).*

Figure S6.4 shows the 9 GHz EPR spectrum of the reference sample taken at time 0. The three-line pattern of the nitroxide radical is visible, however the spectrum is dominated by a broad band, which can be related to spin-spin interactions between the labels. The Lorentzian-derivative fitting of the broad band (red) tells us that more than 95 % of the labels are subject to such interactions. The R1CP-2 was completely labeled, therefore, there was no diamagnetic dilution in the sample. If macromolecular aggregates are formed, confining the labels to distances in the range of 5 Å[202,203], such an exchange-broadened signal is expected. The measurements done with Aβ WT show the same results (spectra not shown), showing that the interaction between inhibitor and Aβ cannot be detected under these conditions.