Supplementary Information

A Catalytic Antioxidant for Limiting Amyloid-Beta Peptide Aggregation and Reactive Oxygen Species Generation

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Experimental

All common chemicals were purchased from Aldrich and used without further purification. The compound Iron(III) 2,17-bis-sulfonato-5,10,15-tris(pentafluorophenyl)corrole (**FeL1**) was synthesized as reported.[1] The $A\beta_{1–16}$, $A\beta_{17–40}$ and $A\beta_{1–42}$ peptides were purchased from 21st Century Biochemicals (Marlborough, MA, USA), Pepmic Co., Ltd (Suzhou, China) and Cellmano Biotech Limited (Hefei, China), and monomerized before use according to a reported procedure.[2] $A\beta_{1–16}$ was dissolved in double distilled H_2O (ddH_2O), while $A\beta_{1–42}$ and $A\beta_{17–40}$ were dissolved in DMSO and ddH_2O in a 1:1 mixture, unless stated otherwise. The stock peptide solution concentration was determined by absorbance with the use a Thermo Nicolet UV nanodrop and an extinction coefficient of 1410 and 1450 $M^{-1}cm^{-1}$ at 280 nm, for $A\beta_{1–16}$, and $A\beta_{1–42}$ respectively.[3] A Bradford assay (BioRad) was used to determine the concentration of $A\beta_{17–40}$. Human serum albumin (HSA) was as a lyophilized powder from Sigma Aldrich. An Amplex red catalase kit was purchased from Thermo Fisher Scientific. ^{1}H NMR spectra were recorded on a Bruker AV-600 instrument. Electronic spectra were obtained on a Cary 5000 spectrophotometer. All ROS assays were measured using a Synergy 4 Multi-Detection microplate reader from BioTek. TEM images were obtained using an OSIRIS FEI scanning TEM (STEM) operating at 200 kV. X-band (9.4 GHz) EPR spectra were collected using a Bruker EMX plus spectrometer equipped with a Premium X microwave bridge and HS resonator. Samples were run in 4 mm outer-diameter quartz tubes.

UV-Vis Binding Assay of FeL1 with 1-MeIm and Aβ

$A\beta_{1-16}$ was dissolved in 1:1 DMSO/ddH_2O solution. **FeL1** was dissolved in PBS buffer (0.01 M, pH 7.4), and one equiv. of the $A\beta_{1-42}$, $A\beta_{1-16}$ or $A\beta_{17-40}$ was added to **FeL1** and spectra monitored over time. Spectral changes were observed immediately with no further changes for up to 1 hr. The binding of **FeL1** to $A\beta_{1-16}$ in the presence of Cu(II) was also investigated by UV-Vis. $CuCl_2$ was dissolved in PBS (0.01 M, pH 6.3) buffer and added at 0.9 equiv. to the $A\beta_{1-16}$ peptide (30 μM) and incubated for 10 min to ensure binding. 1 equiv. of **FeL1** was then added to the solution of the Aβ-Cu(II) species. Adding the reagents in reverse order (**FeL1** and then Cu(II)) resulted in the same spectral features. The spectral changes associated with 1-methylimidazole (1-MeIm) binding to **FeL1** were evaluated in PBS buffer (0.01 M, pH 7.4). To a solution of **FeL1**, 1-MeIm was added in aliquots (up to 150 equiv.) until no further spectral changes were observed. A variable pH titration was performed in ddH_2O with **FeL1** and 2 equiv. of 1-MeIm to determine a binding constant. Data were collected from 200-900 nm, and small aliquots of 0.1 M NaOH were titrated into the solution to adjust the pH. 25 UV-Vis spectra were collected in the pH 2.6-10.3 range. Spectral data were analyzed using HypSpec (protonic Software UK)[4], using the pKa value of 7.0 for 1-MeIm.[5] Metal speciation

plots were created using the HySS2009 program (protonic Software, UK).[4] The binding constant of $A\beta_{1-16}$ with **FeL1** was obtained in a similar fashion to 1-MeIm, however a 1:1 concentration ratio of the peptide and **FeL1** was employed. Data were collected from 200-900 nm, and small aliquots of 0.1 M NaOH were titrated into the solution to adjust the pH; 37 UV-Vis spectra were collected in the pH 3.1-11.5 range. Spectral data were analyzed using HypSpec (protonic Software UK)[4], using reported pKa values for $A\beta_{1-16}$.[6]

^{1}H NMR Binding Assay of $A\beta_{1-16}$ Peptide to FeL1

Deuterated PBS (0.01 M, pH 7.4) buffer was prepared by removal of water by vacuum drying of PBS buffer and dissolving the powder in D_2O. $A\beta_{1-16}$ and **FeL1** were dissolved in deuterated PBS (0.01 M, pH 7.4) buffer, and the ^{1}H NMR spectra of $A\beta_{1-16}$ alone followed by additions of **FeL1** in 0.1 and 0.25 equiv. were acquired.

Mass Spectrometry of Binding of $A\beta_{1-16}$ Peptide to FeL1 and Cu(II)

ESI-TOF-MS experiments were performed on an Agilent 6130 mass spectrometer connected to an Agilent 1260 HPLC system. Samples were analyzed by direct infusion (1-4 μL) of analyte into a mobile phase of 1:1 water:acetonitrile containing 5 mM ammonium acetate (pH unmodified), flowing at 0.3 mL/min and maintained at 30 °C. All components of the mobile phase were mass spectrometry grade. Nitrogen drying gas was 250 °C, 5 L/min with a nebulizing pressure of 15 psig. Voltages were: capillary 3 kV, fragmentor 175 V, skimmer 30 V, octopole 250 V. Samples were prepared as ~4 mg/mL of total protein ($A\beta_{1-16}$ and/or HSA) in ammonium carbonate (0.02 M, pH 9) buffer with 0 or 1 equivalents of $CuCl_2$ and/or **FeL1**.

EPR Analysis

EPR measurements were conducted using a Bruker EMXplus spectrometer at 20 K at X-band with a PremiumX microwave bridge and HS resonator and a Bruker ER 4112HV helium temperature-control system and continuous-flow cryostat, the Bruker cryostat allows for reproducible sample placement with minimal variance in Q-factor. As a result, by using identical spectroscopic parameters and automatic tuning of the spectrometer it was possible to compare the intensities of the different Cu(II) species in solution. The final concentration of $A\beta_{1-16}$ was 550 μM, and in order to ensure a predominance of peptide-bound metal species, Cu(II) and **FeL1** concentrations were 500 μM. Samples were prepared in 270 μL of PBS (0.05 M, pH 7.4), incubated at room temperature for 30 min, and then mixed with 30 μL of glycerol (which acted as a glassing agent) immediately before freezing in liquid N_2. The intensity on the Fe(III) EPR signal at 77 K was significantly smaller in comparison to the spectra at 20 K. All spectra were simulated with the Matlab-based program, EasySpin.[7]

Gel Electrophoresis and Western Blotting

$A\beta_{1-42}$ film was dissolved in 1:1 DMSO/ddH_2O to obtain a stock solution with a concentration of approximately 300 μM. Aβ solutions with a concentration of 25 μM were prepared in PBS (0.01 M, pH 7.4) then incubated for 24 h at 37 °C with continuous agitation at 200 rpm to form aggregates in the presence of different concentrations of **L1**, or **FeL1**. Electrophoresis separation of peptide aggregates employed 10-20% Mini-PROTEAN® Tris-Tricine Precast Gels from Bio-Rad, at 100 V for 100 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4 °C, followed by blocking of the membrane in a 3% BSA solution in TBS for 1 hour. The membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-Aβ primary antibody (Biolegends) overnight. After washing 4 × 15 min with TBS buffer, the membrane was incubated in a solution containing the secondary antibody (Horseradish peroxidase, Caymen Chemicals) for 3 hours. Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate kit was used to visualize the Ab species using a FUJIFILM Luminescent Image Analyzer (LAS-4000).

Transmission Electron Microscopy (TEM)

Samples were prepared from the Western blot assay after the 24 hour incubation time at 37 °C. TEM grids were prepared following previously reported methods.[8, 9] In order to increase hydrophilicity, the Formvar/Carbon 300-mesh grids (Electron Microscopy Sciences) were glow discharged in a vacuum for 15 seconds. Drops of samples (10 μL) were placed onto a sheet of parafilm and the TEM grid was laid on the drop for 5 minutes. The grid was then placed and immediately removed on the first and second drop of syringe-filtered 5% uranyl acetate, then placed on the third drop to incubate for 1 minute. Excess uranyl acetate was removed using a tissue between drops. The grid was allowed to air-dry for at least 15 minutes. Bright field images were obtained on a FEI Tecnai Osiris STEM at 200 kV.

Catalase Activity Assay

The Amplex red reagent was used in the presence of horseradish peroxidase (HRP) to detect H_2O_2 formation, producing a fluorescent compound, resorufin.[10] **FeL1** was dissolved in 0.1 M Tris-HCl buffer, pH 7.5. A fresh 10 mM stock solution of L-ascorbic acid was prepared in 0.1 M Tris-HCl buffer, pH 7.5 and protected from light until use. The Amplex red assay was performed in quadruplicate in a flat-bottomed clear 96-well plate (Microtest, BD Falcon). A working solution was prepared containing 100 μM Amplex red reagent and 0.4 U/mL HRP, where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. Different concentrations of catalase, from 0 to 1000 mU/mL, where 1 unit is defined as the amount of enzyme that will decompose 1.0 μM of H_2O_2 per minute at pH 7.0 at 25°C, were used to obtain a standard curve. **FeL1** catalase activity was determined for different concentrations of the complex alone and in the presence of $A\beta_{1\text{-}16}$. All compounds were incubated at room temperature with 40 μM H_2O_2 for 30 min, followed by addition of working solution containing Amplex red and HRP and incubated for another 15 min before measuring the absorbance at 570 nm.

Aβ-Cu(II) ROS Production

$A\beta_{1\text{-}16}$ was dissolved in ddH_2O and a fresh 10 mM stock solution of L-ascorbic acid was prepared in PBS buffer (0.01 M, pH 7.4) and kept protected from light until use. **FeL1** was dissolved in PBS buffer (0.01, M pH 7.4) and $CuCl_2$ was dissolved in PBS buffer (0.01 M, pH 6.6). The compounds were dissolved as described here in the following assays, unless stated otherwise.

Ascorbate Assay. The absorbance of an initial concentration of 100 μM of Asc at 265 nm was measured over time at room temperature in a quartz cuvette for each sample. Final concentrations of $A\beta_{1\text{-}16}$ and FeL1 were 10 μM, and Cu(II) 9 μM.

Hydrogen Peroxide Assay. For this assay all components, with the exception of the $A\beta_{1\text{-}16}$, were dissolved in a 0.1 M Tris-HCl buffer, pH 7.5. A similar procedure to the catalase assay was performed using the Amplex red kit, with exception of the addition of H_2O_2 and catalase. The compounds were added to the wells followed by addition of 50 μL of the working solution. L-ascorbic acid was the last component added, initializing the reaction between dioxygen and Cu(II). The formation of resorufin was monitored by absorbance at 570 nm at RT for 45 min.

Coumarin-3-carboxylic Acid Assay. Coumarin-3-carboxylic acid (3-CCA) was used to detect hydroxyl radicals (•OH) produced by Aβ-Cu(II) in the presence of L-ascorbic acid.[11] The CCA assay was conducted in quintuplicate in a flat-bottomed black 96-well plate (Microtest, BD Falcon). 3-CCA was dissolved in PBS buffer (0.01 M, pH 7.4). Cu(II), 3-CCA and L-ascorbic acid had final concentrations of 40 μM, 100 μM and 300 μM, respectively. Production of •OH by Cu(II) was determined in the presence of 1 equivalent of $A\beta_{1\text{-}16}$ and **FeL1**. Reaction of 3-CCA with •OH produces 7-OH-CCA, a fluorescent compound with λ_{ex}: 395 nm and λ_{em}: 450 nm. The reaction was initiated by addition of L-ascorbic acid and was monitored at room temperature for 45 min. Controls of compounds and 3-CCA without L-ascorbic acid showed no significant fluorescence.

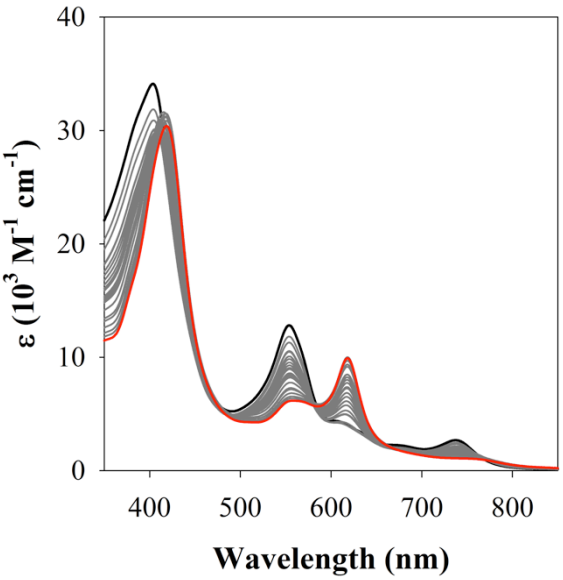

Figure S1: UV-Vis spectra of L-Histidine (L-His) additions to **FeL1** (30 μM, black) in PBS buffer (0.01 M, pH 7.4). Grey lines represent additions of 10 equiv. of L-His up to 700 equiv. shown in red.

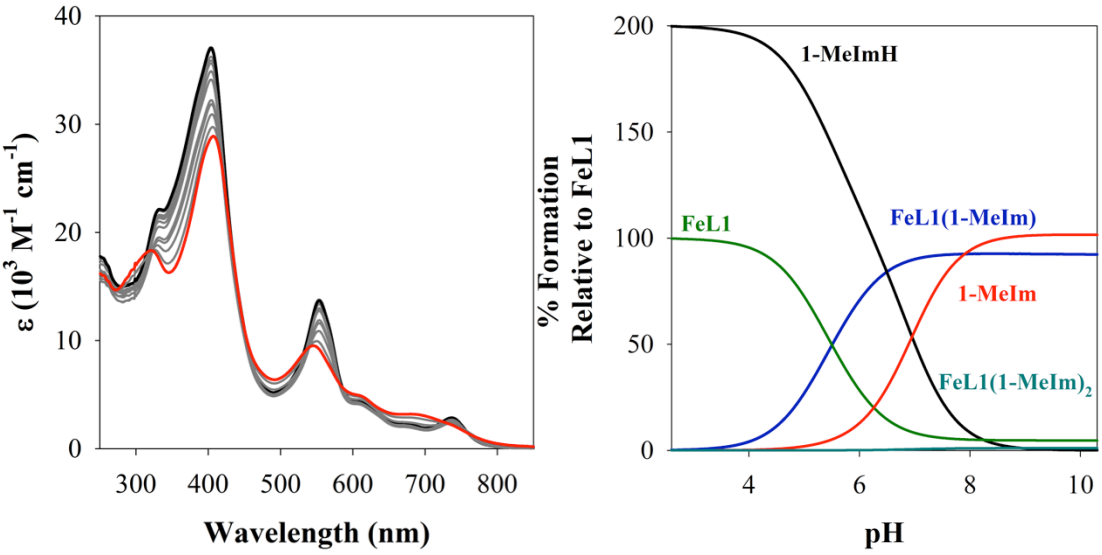

Figure S2: (Left) Variable pH UV-Vis titration of **FeL1** (30 μM) with 1-MeIm (60 μM) from pH 3.4 (black) to pH 10.5 (red). (Right) Using HypSpec and HySS,[12] the variable pH data were fit to a model including **FeL1**(1-MeIm) and **FeL1**(1-MeIm)$_2$,. At pH 7.4 the majority of **FeL1** is bound to 1-MeIm (>99%).

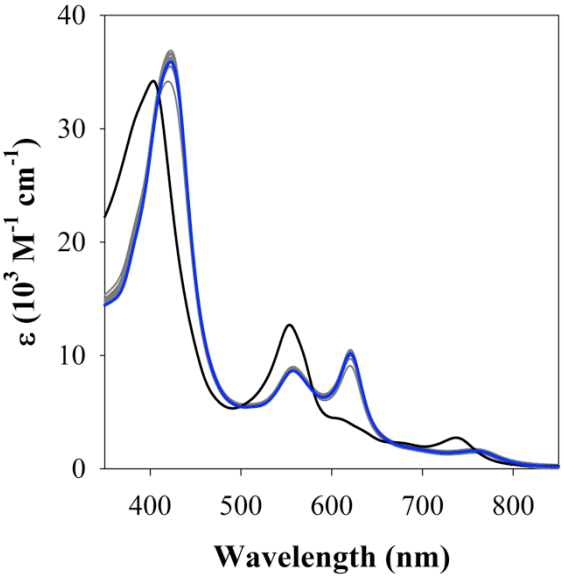

Figure S3: UV-Vis spectra of $A\beta_{1-16}$ titration with **FeL1** (30 μM, black) in PBS buffer (0.01 M, pH 7.4). Grey lines represent additions 1 equiv. of $A\beta_{1-16}$ up to 16 equiv. (blue).

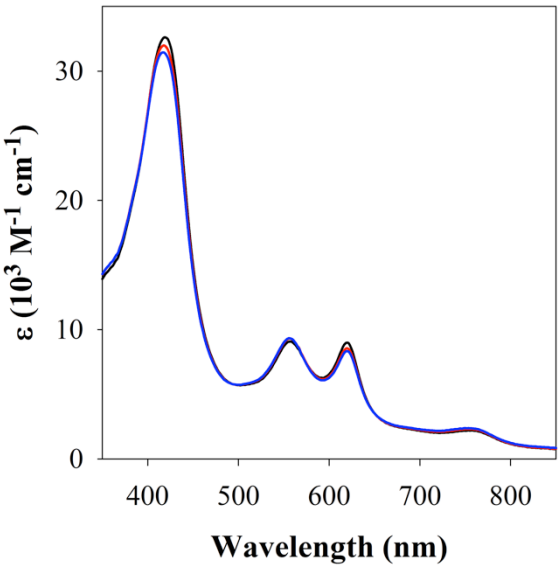

Figure S4: UV-Vis spectra of binding of **FeL1** (30 μM) and Cu(II) (27 μM) to $A\beta_{1-16}$ (30 μM) in PBS buffer (0.01 M, pH 7.4). Order of addition varied to observe any potential changes in binding, where the species **FeL1**-Aβ is shown in black, and the species **FeL1**-Aβ-Cu(II) are shown in red and blue, with **FeL1** or Cu(II) being added first to a solution containing $A\beta_{1-16}$, respectively.

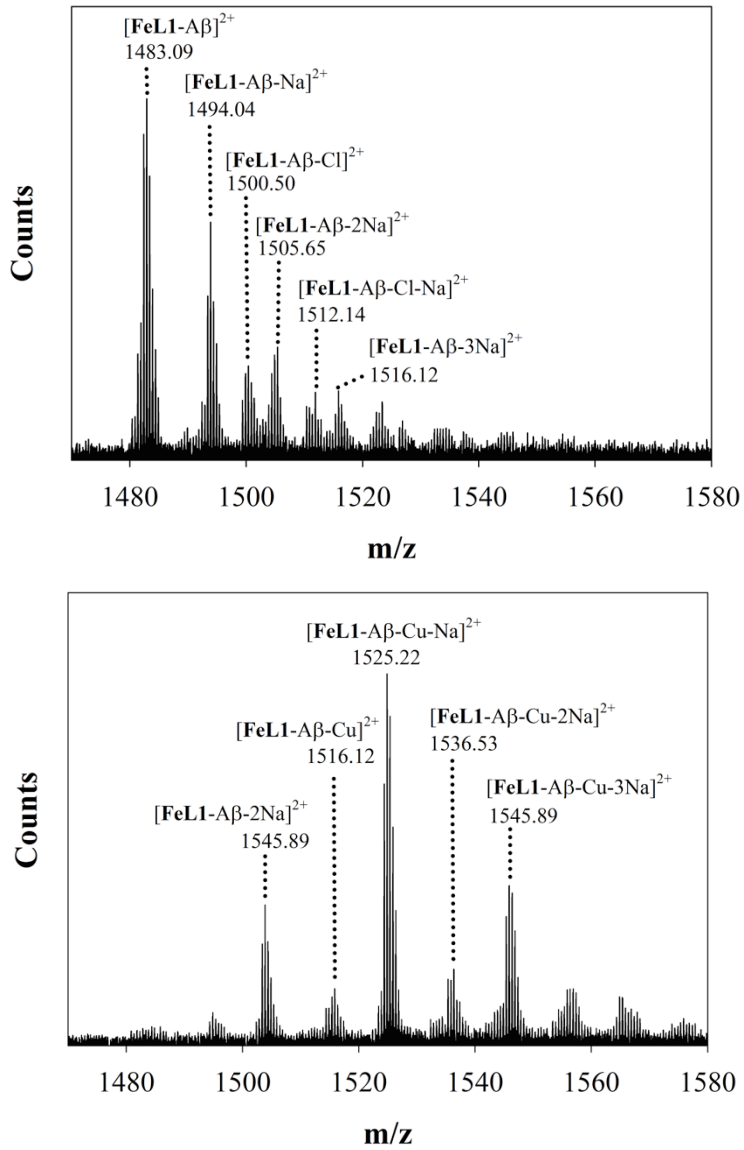

Figure S5: Mass spectrum (ESI-MS) of binding of $A\beta_{1-16}$ to **FeL1** and Cu(II). (Above) **FeL1**-$A\beta_{1-16}$ with its chloride and sodium adducts and (bellow) **FeL1**-$A\beta_{1-16}$-Cu with its sodium adducts. Across all experiments, species were observed as $[\mathbf{FeL1}_{(0-1)}\text{-}A\beta\text{-}Cu_{(0-1)}\text{-}Cl_{(0-1)}\text{-}Na_{(0-6)}]^{+2-3}$. Additionally was observed the dimeric species $[A\beta_2\text{-}Cl_1\text{-}Na_{(0-6)}]^{+3}$ at m/z 1303-1374.

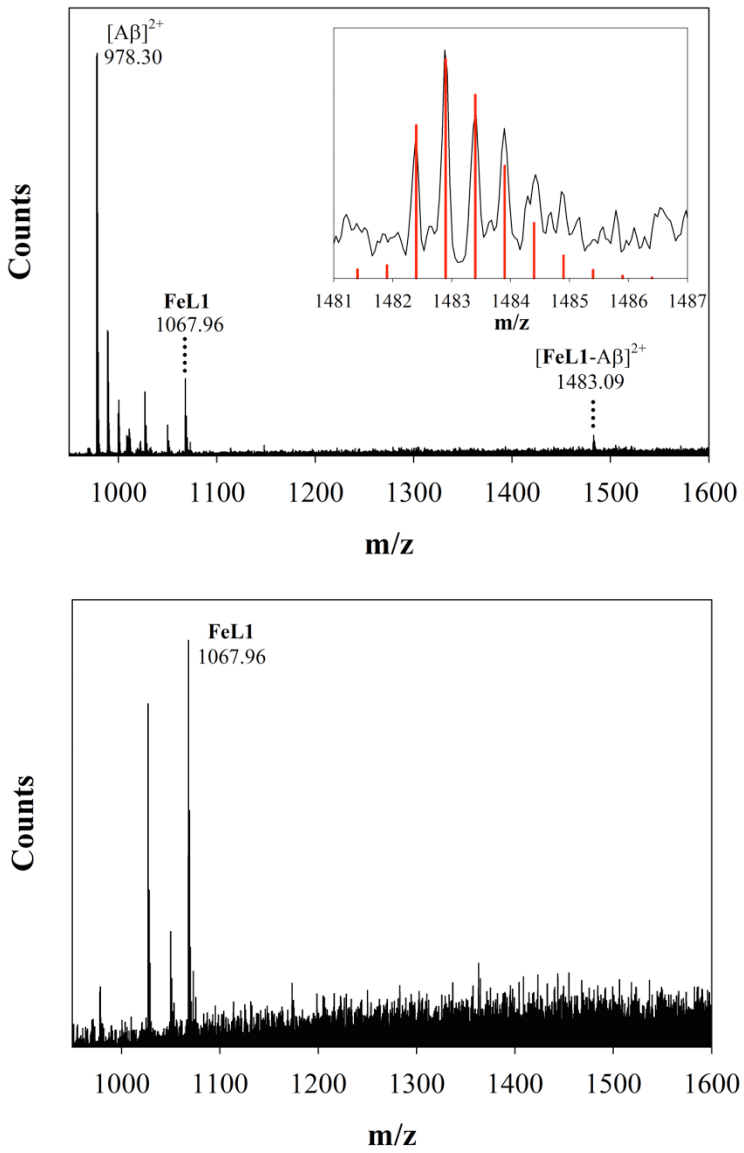

Figure S6: Mass spectrum (ESI-MS) of binding of $A\beta_{1\text{-}16}$ to **FeL1** in the presence of human serum albumin (HSA). **FeL1**-$A\beta_{1\text{-}16}$ and its isotopic pattern inset (above) and free **FeL1** in the presence of HSA (below).

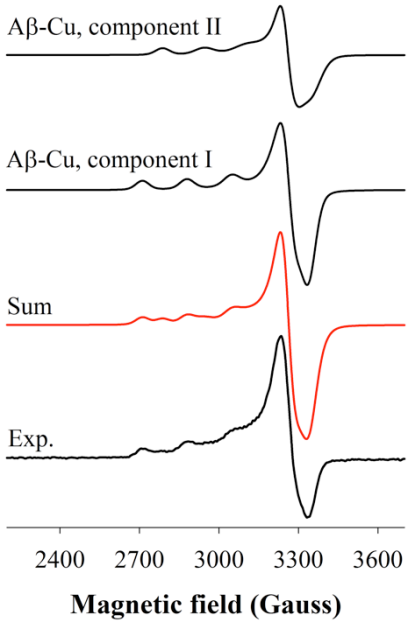

Figure S7: Frozen-solution EPR spectrum (bottom), simulation (red) and spectral deconvolution of Cu(II) in the presence of 1 equiv. of $A\beta_{1\text{-}16}$ and Cu(II) at 20 K. **Conditions:** [$A\beta_{1\text{-}16}$] = 550 μM, [Cu(II)] = 500 μM, in PBS buffer (0.05 M, pH 7.4). **EPR parameters:** frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans.

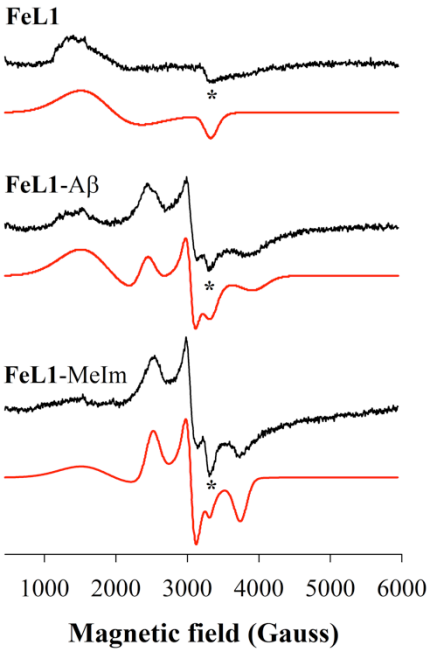

Figure S8: Frozen-solution EPR spectra (black) and simulations (red) collected at 20 K of **FeL1**, **FeL1**-$A\beta_{1\text{-}16}$ and **FeL1**-MeIm. The linewidth for the **FeL1**-MeIm g_2 feature (S = 3/2) is decreased by a factor of 2 relative to **FeL1**. **Conditions:** [$A\beta_{1\text{-}16}$] = 0.55 mM, [**FeL1**] = 0.50 mM, [1-MeIm] = 10 mM in PBS buffer (0.01 M, pH 7.4). **Experimental parameters:** frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans. *The intermediate spin g_2 signal overlaps with an instrument background signal.

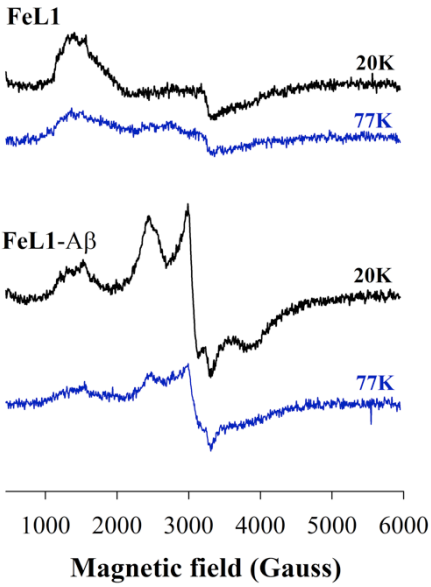

Figure S9: Frozen-solution EPR spectra collected at 20 K (black) and 77 K (blue) of **FeL1** and **FeL1**-$A\beta_{1\text{-}16}$. **Conditions:** [$A\beta_{1\text{-}16}$] = 0.55 mM, [**FeL1**] = 0.50 mM in PBS buffer (0.01 M, pH 7.4). **Experimental parameters:** frequency = 9.38 GHz, microwave power = 2.0 mW, time constant = 40.96 ms, modulation amplitude = 5 G, average of five 1-min scans.

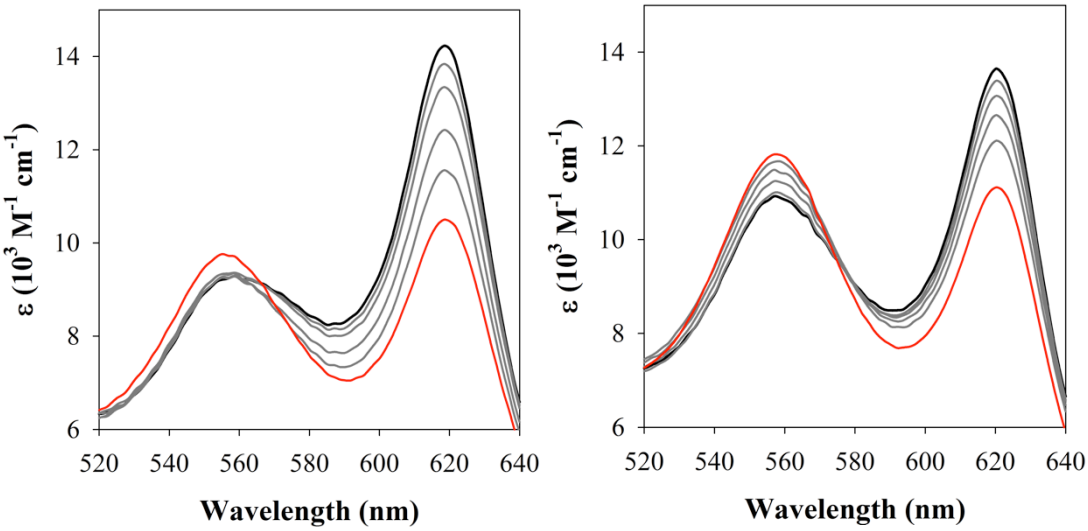

Figure S10: UV-Vis spectra of the temperature dependence of binding of **FeL1** (30 μM) to 1-MeIm (600 μM, left) or $A\beta_{1\text{-}16}$ (30 μM, right) in PBS buffer (0.01 M, pH 7.4). Grey lines represent changes of temperature starting at 10 ˚C (black) to 37 ˚C (red). The spectral changes are reversible and indicate an increase in bis-axial binding to **FeL1** as temperature is lowered.

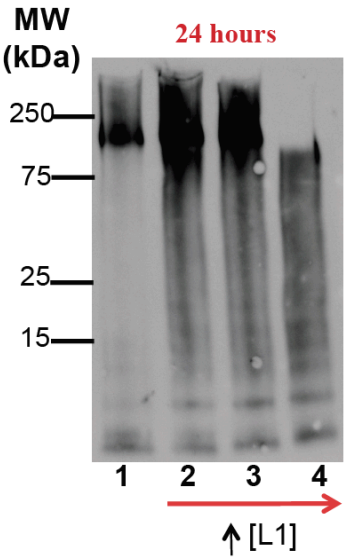

Figure S11: Influence of **L1** on the aggregation profile of $A\beta_{1-42}$. Gel electrophoresis/Western blot of 25 μM $A\beta_{1-42}$ and different concentrations of **L1** in PBS buffer (0.01 M, pH 7.4) after 24 h incubation with agitation at 37 ˚C, using anti-Aβ antibody 6E10. Lane 1: $A\beta_{1-42}$; lane 2: $A\beta_{1-42}$ + **L1** (0.1 eq); lane 3: $A\beta_{1-42}$ + **L1** (0.5 eq); lane 4: $A\beta_{1-42}$ + **L1** (1 eq).

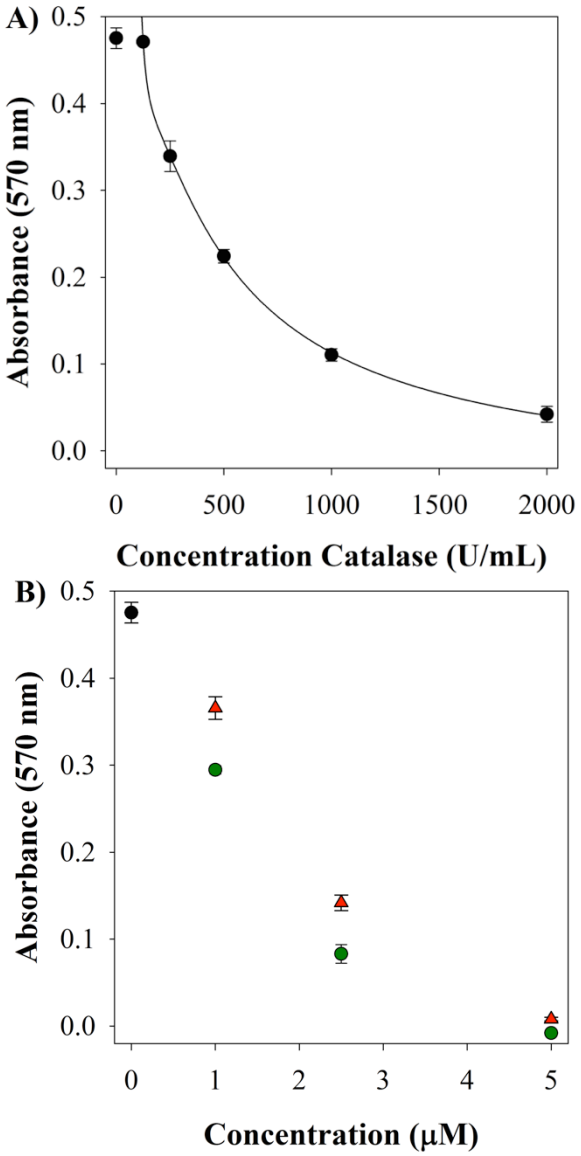

Figure S12: Catalase activity measured by absorbance of the amplex red reagent (λ = 570 nm) in the presence of H_2O_2 (10 μM) after 30 min incubation of catalase enzyme, **FeL1** or **FeL1**-Aβ. **A)** Catalase standard curve, and **B) FeL1** and **FeL1**-Aβ at different concentrations. [$A\beta_{1\text{-}16}$]=[**FeL1**]=1, 2.5, 5 μM ● Control; ▲ **FeL1**; ● **FeL1**-Aβ.

References

1. a) A. Mahammed, H. B. Gray, J. J. Weaver, K. Sorasaenee and Z. Gross, *Bioconjug. Chem.*, 2004, **15**, 738-746; b) A. Mahammed; Z. Gross, *J. Am. Chem. Soc.*, 2005, **127**, 2883-2887; c) A. Kanamori, M.-M. Catrinescu, A. Mahammed, Z. Gross and L. A. Levin, *J. Neurochem.*, 2010, **114**, 488-498.
2. a) R. Sabate, M. Gallardo and J. Estelrich, *Biopolymers*, 2003, **71**, 190-195; b) S. K. Pachahara, N. Chaudhary, C. Subbalakshmi and R. Nagaraj, *J. Pept. Sci.*, 2012, **18**, 233-241.
3. a) L. Guilloreau, S. Combalbert, A. Sournia-Saquet, H. Mazarguil and P. Faller, *ChemBioChem*, 2007, **8**, 1317-1325; b) K. A. Coalier, G. S. Paranjape, S. Karki and M. R. Nichols, *Biochim. Biophys. Acta*, 2013, **1834**, 65-70.
4. P. Gans, A. Sabatini and A. Vacca, *Ann. Chim. (Rome)*, 1999, **89**, 45-49.
5. J. Liu, Z. Wang, A. Levin, T. J. Emge, P. R. Rablen, D. M. Floyd and S. Knapp, *J. Org. Chem.*, 2014, **79**, 7593-7599.
6. T. Kowalik-Jankowska, M. Ruta, K. Wiśniewska and L. Łankiewicz, *J. Inorg. Biochem.*, 2003, **95**, 270-282.
7. S. Stoll; A. Schweiger, *J. Magn. Reson.*, 2006, **178**, 42-55.
8. M. R. Jones, E. L. Service, J. R. Thompson, M. C. P. Wang, I. J. Kimsey, A. S. DeToma, A. Ramamoorthy, M. H. Lim and T. Storr, *Metallomics*, 2012, **4**, 910-920.
9. L. Chiang, L. E. N. Allan, J. Alcantara, M. C. P. Wang, T. Storr and M. P. Shaver, *Dalton Trans.*, 2014, **43**, 4295-4304.
10. M. Zhou, Z. Diwu, N. Panchuk-Voloshina and R. P. Haugland, *Anal. Biochem.*, 1997, **253**, 162-168.

11. M. Jensen, A. Canning, S. Chiha, P. Bouquerel, J. T. Pedersen, J. Østergaard, O. Cuvillier, I. Sasaki, C. Hureau and P. Faller, *Chem. Eur. J.*, 2012, **18**, 4836-4839.
12. L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini and A. Vacca, *Coord. Chem. Rev.*, 1999, **184**, 311-318.