

Cite this: *Dalton Trans.*, 2016, **45**, 15671

How Zn can impede Cu detoxification by chelating agents in Alzheimer's disease: a proof-of-concept study†

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The role of Cu and Zn ions in Alzheimer's disease is linked to the consequences of their coordination to the amyloid- β (A β) peptide, *i.e.* to the modulation of A β aggregation and to the production of Reactive Oxygen Species (ROS), two central events of the so-called amyloid cascade. The role of both ions in A β aggregation is still controversial. Conversely the higher toxicity of the redox competent Cu ions (compared to the redox inert Zn ions) in ROS production is acknowledged. Thus the Cu ions can be considered as the main therapeutic target. Because Zn ions are present in higher quantity than Cu ions in the synaptic cleft, they can prevent detoxification of Cu by chelators unless they have an unusually high Cu over Zn selectivity. We describe a proof-of-concept study where the role of Zn on the metal swap reaction between two prototypical ligands and the Cu(A β) species has been investigated by several complementary spectroscopic techniques (UV-Vis, EPR and XANES). The first ligand has a higher Cu over Zn selectivity relative to the one of A β peptide while the second one exhibits a classical Cu over Zn selectivity. How Zn impacts the effect of the ligands on Cu-induced ROS production and A β aggregation is also reported.

Received 10th June 2016,
Accepted 23rd August 2016
DOI: 10.1039/c6dt02308h

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population with an estimated prevalence of 30 million people worldwide early in the decade.¹ AD is a multi-factorial neurodegenerative disease in which many features are involved. This may be the reason why only symptomatic drugs with limited effect are currently available.² Hence the development of curative treatments is urgently needed. This requires a right understanding of the trafficking between the drug candidate and the biological partners.

In AD, aggregation of the amyloid- β peptide (A β), a 40 to 42 amino-acid residue peptide, forming amyloid plaques has been considered to be a central process and an early event in the pathology, known as the amyloid cascade.^{1,3,4} Indeed, while amyloid plaques are observed post-mortem in AD patients' brains, the monomeric soluble A β peptide is present in healthy patients. Metal ions, mainly Cu(I/II) and Zn(II), have

been related to alteration of the A β aggregation process^{5,6} and both metal ions are bound to the A β peptide within the amyloid plaques.⁷ It is generally assumed that these metal ions are bound to A β under AD conditions, but not in healthy subjects.⁸ This makes zinc and copper potential therapeutic targets, and hence chelators to remove these metal ions have been developed (for recent reviews, see ref. 8–13). A milestone article concerning the use of chelators to target metal ions bound to A β was the work of Cherny *et al.* in 2001.¹⁴ It was shown that the chelator clioquinol (5-chloro-7-iodo-quinolin-8-ol) inhibits the accumulation of A β in AD model mice. Later the same group showed that a clioquinol derivative named PBT2 also improved the cognitive performance in AD model mice.¹⁵ More recent studies on the impact of chelators on AD model mice include the studies of bis-8-aminoquinoline derivatives¹⁶ and of the so-called metamorphosizers.¹⁷ However, these seminal studies did not address the question of which metal is targeted by the chelators. Until now, whether the key target is Cu(I/II) or Zn(II) or both is still uncertain, because the effect of these metal ions on the A β aggregation process is dependent on conditions. Indeed, while the impact of Cu(II) and Zn(II) on the aggregation of A β and cell toxicity has been studied by different groups and reported in a multitude of publications (see *e.g.* ref. 18–22 and for recent reviews, see ref. 5 and 23), there is no clear trend as results are dependent on conditions (concentration, stoichiometry, pH, peptide

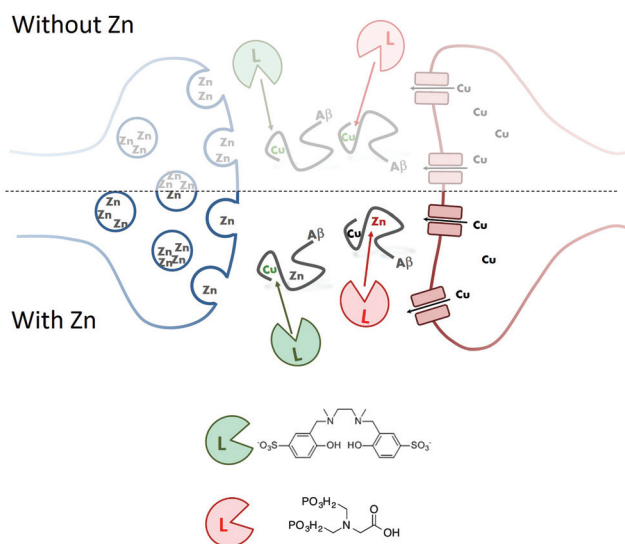
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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c6dt02308h

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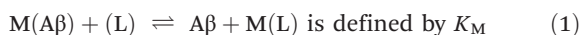




Scheme 1 Top: Classical strategy where Zn is not considered corresponding to a situation with low biological relevance. Bottom: Present strategy where Zn is considered in the exchange reaction. Cu removal by a ligand with $S_L > S_{A\beta}$ is shown in green and by a ligand with $S_L < S_{A\beta}$ in red. In the present study, the Cu : Zn : A β : L stoichiometry was fixed to 1 : 1 : 1 : 1. The complete set of equations describing this situation is detailed in the ESI.†

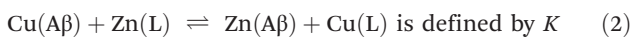
A schematic situation within the synaptic cleft is shown in Scheme 1 and is described by the equations and notations given below (with M = Cu(II) or Zn(II)).

Without Zn:



where $K_M = \frac{K_L^M}{K_{A\beta}^M}$ and K_L^M corresponds to the $M + L \rightleftharpoons M(L)$ equilibrium

With Zn:



where $K = \frac{K_L^{Cu} K_{A\beta}^{Zn}}{K_{A\beta}^{Cu} K_L^{Zn}} = \frac{S_L}{S_{A\beta}}$

where $S_L = \frac{K_L^{Cu}}{K_L^{Zn}}$ and corresponds to $Cu(L) + Zn \rightleftharpoons Zn(L) + Cu$ (3)

Results and discussion

In the present communication, we illustrate the importance of Zn(II) interference into Cu(II) targeting by ligands, including removing from A β and the impact on associated events, *i.e.* ROS production and A β aggregation. As a prototypical ligand with an appropriate Cu(II) over Zn(II) selectivity ($\log(S_{L_2}) = 7.7$, *i.e.* $S_{L_2} > S_{A\beta}$)^{44,46} we have used a water-soluble Schiff base derivative noted as L₂ (in green in Scheme 1) and compare its

properties to those of a control ligand noted as Lc (in red in Scheme 1) having an appropriate Cu(II) affinity ($\log(K_{Cu}) = 2.4$) but an inappropriate selectivity ($\log(S_{Lc}) = 2.0$, *i.e.* $S_{Lc} < S_{A\beta}$).⁴⁹ The peptides used here are either the N-terminal part of the full-length peptide encompassing the residues involved in metal ions coordination (sequence DAEFRHDSGYEVHHQK noted as A β 16) and considered as a valuable and more soluble model of the A β 40^{3,50} or the full length A β 40 peptide, noted as A β in the following. This is a proof-of-concept study in the sense that the ligands used are not suitable for direct use *in vivo* due to a probable bad Blood Brain Barrier (BBB) penetration ability. Indeed, the main objective is to illustrate the impact of Zn(II) in Cu(II) removal from A β , an issue that has been overlooked. It might seem evident to anticipate the outcome of the experiments, since all the affinities of the four complexes are known. However, Zn(II) and Cu(II) bind to several binding sites in fast equilibrium and the main Zn(II) and Cu(II) sites do partially overlap.⁵¹ This is not a classical situation and it is worth confirming the expected results by a set of complementary experiments. In addition, the approach developed here would also be very useful for other ligands with potential as drug candidates when the affinity constants cannot be determined (for instance due to solubility issue).

First, Cu(II) removal from A β in the presence of Zn(II) has been monitored for both L₂ and Lc ligands by UV-Vis (Fig. 1, left and Fig. S1, S2†) and EPR (Fig. 1, right and Fig. S3†). UV and EPR signatures show that: (i) for L₂, the Cu(II) ion is chelated by the ligand and not by the peptide (compare traces

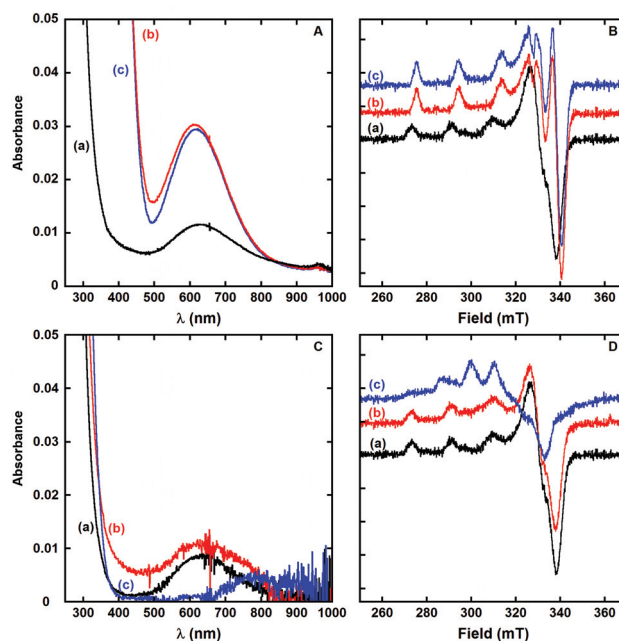


Fig. 1 UV-Vis absorption spectra (left) and EPR signatures (right) of (a) Cu(A β 16), (b) Cu(A β 16) + Zn(L) and (c) Cu(L), L = L₂ (top, panels A and B) or Lc (bottom, panels C and D). For UV-vis experiments: [A β 16] = [L] = 0.1 mM, [M] = 0.1 mM (M = Cu(II) or Zn(II)), [hepes] = 0.1 M, pH 7.1, T = 25 °C, l = 1 cm; for EPR experiments: [A β 16] = [L] = [Zn] = 0.20 mM, [Cu] = 0.18 mM, [hepes] = 50 mM, pH 7.1, T = 110 K.



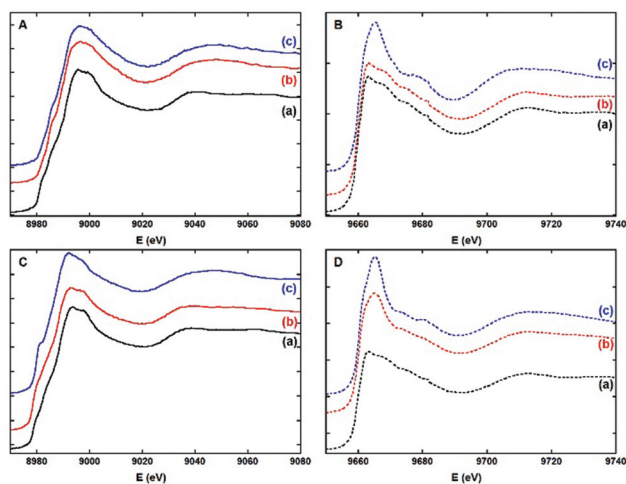


Fig. 2 Normalized Cu (left, panels A and C) and Zn (right, panels B and D, dotted lines) K-edges X-ray absorption near edge structure (XANES) spectra of (a) M(A β 16) (M = Cu(II) or Zn(II)), (b) Cu(A β 16) + Zn(L) and (c) M(L), (M = Cu(II) or Zn(II), L = L₂ (top, panels A and B) or Lc (bottom, panels C and D); [A β 16] = [L] = 1.0 mM, [M] = 1.0 mM, [hepes] = 0.1 M, pH 7.1, T = 20 K.

(b) to traces (a) and (c) in Fig. 1, top) and (ii) for Lc, Cu(II) mainly remains bound to A β (compare traces (b) to traces (a) and (c) in Fig. 1, bottom). To confirm these conclusions by probing both metal centres, XANES (X-ray Absorption Near Edge Structure) measurements were performed. This spectroscopy is the most appropriate: (i) Zn(II) is silent in most of classical techniques and (ii) Cu and Zn K-edges are close enough to be recorded during the same beam-time session. The Cu- and Zn-edges XANES signatures show that: (i) for L₂, the Cu(II) is bound to the ligand (compare trace (b) to traces (a) and (c) in Fig. 2, panel A) and the Zn(II) to the A β peptide (compare trace (b) to traces (a) and (c) in Fig. 2, panel B). This is evidence of the complete metal exchange between Cu(A β) and Zn(L₂) and (ii) for Lc, the Cu(II) remains mainly bound to the A β peptide (compare trace (b) to traces (a) and (c) in Fig. 2, panel C) and Zn(II) to the ligand (compare trace (b) to traces (a) and (c) in Fig. 2, panel D). This is in line with the absence of metal exchange between Cu(A β) and Zn(Lc), although Cu(II) can be removed from A β by the control ligand Lc in the absence of Zn(II) (Fig. S1–S3[†]). Note that the results described here are also obtained when Zn(II) is pre-incubated with Cu(A β) instead of the ligand.

The ability to stop the Cu(A β) ROS production is a prerequisite of any Cu(II) chelator in the context of oxidative stress linked to AD. Such ROS production can be studied by proven methods (Scheme S3[†])^{35,44,52} that rely either on the UV monitoring of consumption of ascorbate (Asc) (Fig. 3) or on the detection of the fluorescent 7-OH-CCA formed by a reaction between the HO \cdot produced and the 3-CCA (Fig. S5[†]). Fig. 3, curves (a), (b) and (b') show the ascorbate consumption by Cu, Cu(A β) and Cu,Zn(A β) species. In the presence of A β , regardless the co-presence of Zn(II), Asc consumption is decreased com-

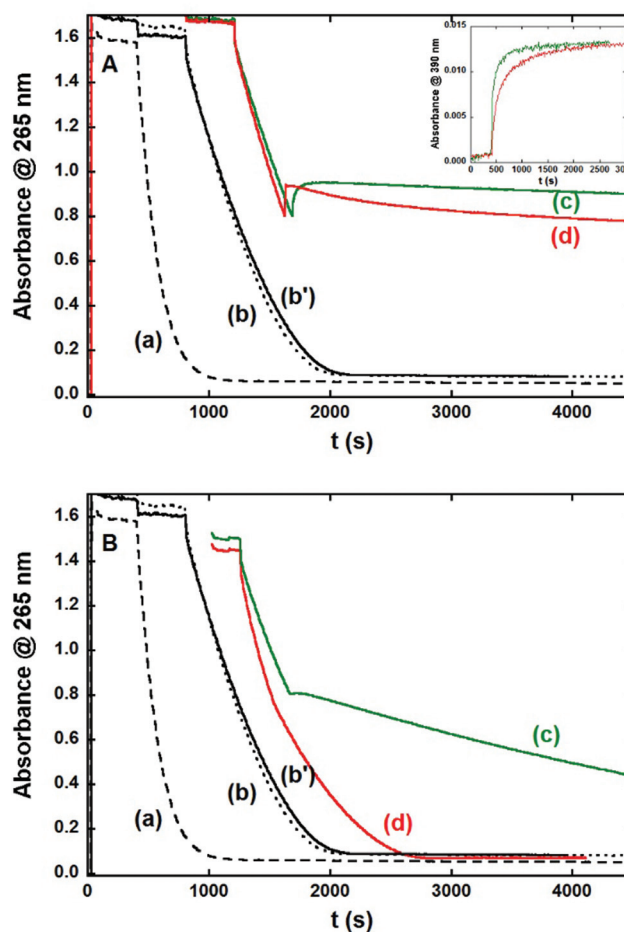


Fig. 3 Ascorbate consumption as a function of time. Time of experiments starts has been arbitrarily shifted for the sake of clarity. (a) Cu; (b, b') Cu(A β) or Cu,Zn(A β), (c) Cu(A β) + L, (d) Cu(A β) + Zn(L), avec L = L₂ (top, panel A) and L = Lc (bottom, panel B). Ascorbate is added as the first reactant. The ligands are added when the absorbance at 265 nm \approx 0.8. [A β] = [L] = 12 μ M, [Zn] = 12 μ M; [Cu] = 10 μ M, [Asc] = 100 μ M, [hepes] = 0.1 M, pH 7.1, T = 25 $^{\circ}$ C. Inset: monitoring of the Cu(L₂) complex formation during the ascorbate consumption experiments corresponding to curves (c) (green line) and (d) (red line).

pared to free Cu(II), as previously observed and discussed.^{35,44} Curves (c) in panels A and B recorded in the absence of Zn(II) show that L₂ (panel A) is able to stop Asc consumption by Cu(A β) while Lc (panel B) can slow it down. Note that just after the addition of the L₂ (curve (c), panel A), there is an increase in the absorbance due to the formation of the Cu(L₂) complex that absorbs at 265 nm, the wavelength where the Asc consumption is monitored. Curves (d) in panels A and B show that in the presence of Zn(II), L₂ (panel A) is able to stop Asc consumption by Cu,Zn(A β) whereas Lc (panel B) has no impact on it, the same trend being observed if Zn(II) is first incubated with the ligand instead of Cu(A β) (Fig. S6[†]) and if Asc is added in the last chemical reaction step (Fig. S7[†]). Note that the Asc consumption experiments are confirmed by the CCA experiments (Fig. S5[†]). In the case of L₂, the arrest of Asc consumption is immediate while for Zn(L₂) a lag phase does



exist during which there is a weak Asc consumption. After this lag phase, slopes of the Asc consumption are similar for L_2 and $Zn(L_2)$. This indicates that the pre-loading of L_2 with $Zn(II)$ slightly slows down the arrest of Asc consumption in line with a slowdown of $Cu(II)$ removal from $A\beta$ by $Zn(L_2)$ compared to L_2 , as monitored by formation of the $Cu(L_2)$ complex (inset in Fig. 3, panel A). In summary, these results show that L_2 can arrest ROS production of $Cu(A\beta)$ even in the presence of $Zn(II)$, whereas L_c can slow it down only in the absence of $Zn(II)$. This is linked to the ability of the $Zn(L_2)$ but not of the $Zn(L_c)$ to remove $Cu(II)$ from $Cu(A\beta)$. In addition, in the course of this experiment, kinetic effects appear when the L_2 ligand is preloaded with $Zn(II)$, although not problematic.

Finally, the impact of L_2 or $Zn(L_2)$ on the $Cu(II)$ modulated $A\beta$ aggregation was monitored by using the classical amyloid reporter fluorophore Thioflavin T (ThT) (Fig. 4, top). Under the present conditions, aggregation of $A\beta$ in the absence or presence of $Zn(II)$ is both characterized by sigmoid-like curves (curves (a) and (d), Fig. 4, top), with a first phase corresponding to nucleation, a second one to the elongation and the third one to the plateau. However, the two curves have different kinetic profiles ($t_{1/2} \approx 50$ h for apo- $A\beta$ and $t_{1/2} \approx 100$ h for $Zn(A\beta)$), where $t_{1/2}$ is the time where the ThT fluorescence equals half the value of the maximum fluorescence. In addition, the maximal ThT fluorescence value is twice as higher for the apo-peptide compared to its Zn complex, but

numerous fibrils are detected by AFM for both systems (Fig. 4, bottom), suggesting that $A\beta$ and $Zn(A\beta)$ both form amyloid fibrils but with different morphologies and ThT interactions. The $Cu(A\beta)$ aggregation kinetic curve exhibits a less defined process leading to a weaker maximal fluorescence and a longer nucleation phase ($t_{1/2} \approx 120$ h), in line with the detection of oligomers and protofibrils by AFM. When performed at the beginning of the aggregation process, addition of L_2 or $Zn(L_2)$ on $Cu(A\beta)$ induces the formation of apo-type fibrils or Zn-type fibrils, respectively (Fig. 4, top, curves c and e). This indicates $Cu(II)$ removal from $A\beta$ or $Cu(II)$ and $Zn(II)$ exchange between $A\beta$ and L_2 , respectively. When L_2 or $Zn(L_2)$ is added later on, similar trends are observed only if addition is done before the elongation phase (Fig. S8[†]). Else if L_2 or $Zn(L_2)$ addition is performed on the plateau phase, no modification of the ThT fluorescence is observed although $Cu(II)$ is readily removed from the fibrils as probed by UV-Vis (Fig. S9[†]). Deeper investigations are foreseen to delineate the impact of adding the chelator after the formation of the fibrils regardless the presence of $Zn(II)$ and are thus beyond the scope of the present study.

Concluding remarks

In the present study, we have demonstrated the importance of taking $Zn(II)$ into account when designing $Cu(II)$ chelators against AD. The impact of $Zn(II)$ on the ability to remove $Cu(II)$ from $A\beta$ and to impede related effects on ROS production and peptide aggregation have been investigated. It was shown that in addition to other requirements, such as being able to redox silence the $Cu(II)$ ions and cross the BBB, the ligand has to display a high $Cu(II)$ over $Zn(II)$ selectivity (*i.e.* higher than the one of $A\beta$). This condition has been overlooked in the literature and is not so easy to satisfy because $A\beta$ is itself very selective for $Cu(II)$ compared to $Zn(II)$, which is linked to the different binding sites adopted by the two metal centres when bound to $A\beta$. This suggests that several chelators designed and used in this context (Tables 1 and S1[†]) would chelate only $Zn(II)$ in a synapse, where the high $Zn(II):Cu(II)$ stoichiometry will enhance the effects of the concept described in the present work with a 1:1 $Cu(II):Zn(II)$ ratio. Indeed, the stoichiometric ratio used here represents “the least unfavourable” condition. This is illustrated by the loss of the arrest of ROS production by the L_2 ligand in the presence of five equivalents of $Zn(II)$ while in the presence of one equivalent of $Zn(II)$ the L_2 was able to completely stop the ROS production (Fig. S6 and S7[†]).

Taking $Zn(II)$ into account when designing a $Cu(II)$ chelator is a first step towards studies under biologically pertinent conditions, and on-going studies include additional impact of pH, the presence of amino-acids and of other ions to be as close as possible to the biological medium. In addition, in the course of the ROS experiments shown here, kinetic issues appear. Indeed, removal of $Cu(II)$ by L_2 preloaded with $Zn(II)$ is slower than that for the ligand only when the $Cu(A\beta)$ is producing ROS. In the case of the L_2 that was not a problem, but similar

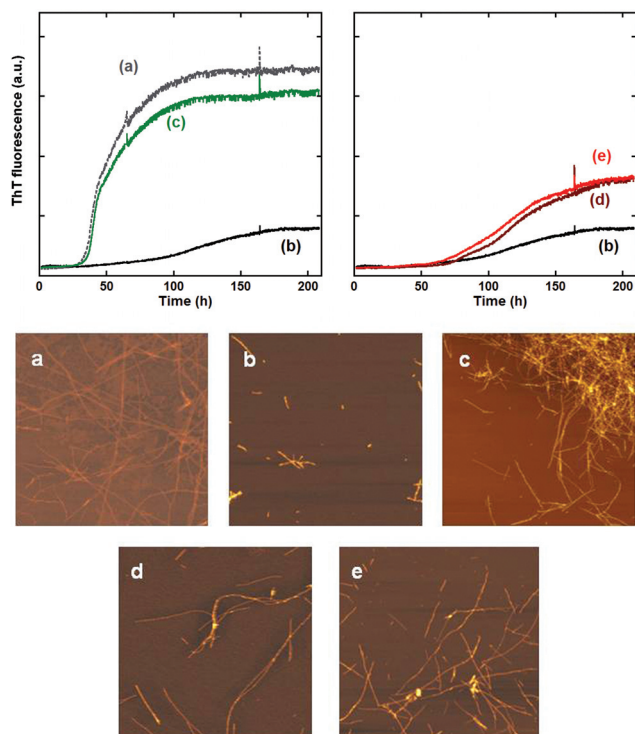


Fig. 4 Top: Kinetic measurement of $A\beta$ aggregates formation using ThT fluorescence. Left: (a) $A\beta$; (b) $Cu(A\beta)$ and (c) $Cu(A\beta) + L_2$; right: (d) $Zn(A\beta)$; (e) $Cu(A\beta) + Zn(L_2)$. $[A\beta] = [L_2] = [L_2-Zn] = 20 \mu M$, $[Cu] = 18 \mu M$, $[phosphate\ buffer] = 0.05\ M$, $pH\ 7.1$, $T = 37\ ^\circ C$. Bottom: AFM images corresponding to ThT curves (a–e), $5\ \mu m \times 5\ \mu m$ pictures.



kinetic effects could preclude the arrest of ROS production in the case of other ligands.

The proof-of-concept shown here for Cu(II) can also apply (i) for Cu(I) chelation, a quite unexplored target,⁵² and (ii) for ligands with a redistribution ability. The capacity to re-distribute the Cu(I/II) ions rather than to eliminate them from A β seems important since it has been proposed that metal deregulation rather than metal overload is a key parameter in AD.^{8,23,53} Strategies to go further towards potential therapeutic applications include: (i) improving the BBB penetration ability, for instance by addition of sugar moieties,^{11,39} or (ii) moving towards multi-functional ligands able to target specific aggregation states, for instance.^{11,54} Lastly, it is worth mentioning that most if not all amyloid related pathologies (such as Parkinson, ALS, *etc.*) also involved several metal ions (Fe, Cu and Zn) with various toxicities.⁵⁵ Hence the metal ions selectivity of a chelator relative to peptides or proteins of interest and described here for Cu(II), Zn(II) and A β is also relevant for other biomolecules and metal ions involved in these diseases.

Experimental section

Chemicals

Reagents were commercially available and were used as received.

Hepes buffer (sodium salt of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) was bought from Fluka (bioluminescence grade).

Phosphate buffer was prepared from K₂HPO₄ and KH₂PO₄ bought from Fluka.

The Cu(II) and Zn(II) sources were Zn(SO₄)(H₂O) and Cu(SO₄)(H₂O)₅ and purchased from Sigma.

A stock solution (5 mM) of ascorbate was prepared in Milli-Q water at room temperature just before beginning the experiment. Because ascorbate degrades quickly, a fresh solution was prepared each day.

A stock solution of coumarin-3-carboxylic acid (CCA, 5 mM) was prepared in phosphate buffer (500 mM, pH 7.1) at room temperature. The stock solution was stored at 4 °C.

A stock solution of Thioflavin T (ThT) at 250 μ M was prepared in water without any further purification with ThT bought from Acros Organics.

Peptides

A β 16 peptide (sequence DAEFRHDSGYEVHHQK and referred to as A β 16) and A β 40 (sequence DAEFRHDSGYEVHHQK LVFFAEDVGSNKGAIIGLMVGGVV and referred to as A β) were bought from GeneCust (Dudelange, Luxembourg) with purity grade >98%.

Stock solutions of the A β 16 peptide were prepared by dissolving the powder in Milli-Q water (resulting pH \sim 2) at approx. 10 mM. Peptide concentration was then determined by UV-visible absorption of Tyr10 considered as free tyrosine (at pH 2, ($\epsilon_{276} - \epsilon_{296}$) = 1410 M⁻¹ cm⁻¹). Stock solutions of the

A β 40 peptide were prepared by dissolving the powder in 50 mM NaOH at approx. 3 mM. Peptide concentration was then determined by UV-visible absorption of Tyr10 considered as free tyrosine (at pH 13, ($\epsilon_{296} - \epsilon_{360}$) = 2400 M⁻¹ cm⁻¹).⁵⁶ The solutions were diluted down to the appropriate concentration in the peptide. All pH values are given with a \pm 0.2 pH unit error.

Monomerization of A β 40. After preparation of the stock solution (see above), A β 40 peptide is monomerized by FPLC, with a Superdex 75 column. 500 μ L of the A β 40 peptide at \sim 3 mM are injected. 15 mM NaOH eluent is used, at 1 mL min⁻¹. The peptide is detected at 293 nm and at a retention time of approx. 10 min. All the fractions corresponding to the peptide are collected and their concentrations are determined by UV-visible spectroscopy.

X-ray absorption spectroscopy

Cu(II) and Zn(II) K-edge XANES (X-ray Absorption Near Edge Structure) spectra were recorded at the BM30B (FAME) beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).⁵⁷ The storage ring was operated in 7/8 + 1 mode at 6 GeV with a 200 mA current. The beam energy was selected using an Si(220) N₂ cryo-cooled double-crystal monochromator with an experimental resolution close to that which was theoretically predicted (namely \sim 0.5 eV FWHM at the Cu and Zn energy).⁵⁸ The beam spot on the sample was approximately 300 \times 100 μ m² (H \times V, FWHM). Because of the low Cu(II) and Zn(II) concentrations, spectra were recorded in fluorescence mode with a 30-element solid state Ge detector (Canberra) in frozen liquid cells in a He cryostat. The temperature was kept at 20 K during data collection. The energy was calibrated with Cu and Zn metallic foils, such that the maximum of the first derivative was set at 8979 and 9659 eV. XANES Cu(II) data were collected from 8840 to 8960 eV using 5 eV step of 2 s, from 8960 to 9020 eV using 0.5 eV step of 3 s, and from 9020 to 9300 eV with a *k*-step of 0.05 \AA^{-1} and 3 s per step. XANES Zn(II) data were collected from 9510 to 9630 eV using 5 eV step of 3 s, from 9630 to 9700 eV using 0.5 eV step of 3 s, and from 9700 to 10 000 eV with a *k*-step of 0.05 \AA^{-1} and 3 s per step. For each sample, at least three scans recorded on different spots were averaged and spectra were background-corrected by a linear regression through the pre-edge region and a polynomial through the post-edge region and normalized to the edge jump. XANES samples were prepared from the stock solution of peptide, ligands and metallic ions diluted down to approx. 1.0 mM in a buffered solution. Samples were frozen in the sample holder after addition of 10% glycerol as a cryoprotectant and stored in liquid nitrogen until use. Cu(II) photoreduction was controlled by recording successive scans at the same spot. It was considered that during the first 20 minutes of recording the photoreduction is insignificant.

Electron paramagnetic resonance

Electron Paramagnetic Resonance (EPR) data were recorded using an Elexsys E 500 Bruker spectrometer, operating at a microwave frequency of approximately 9.5 GHz. All spectra



were recorded using a microwave power of 20 mW across a sweep width of 150 mT (centred at 310 mT) with a modulation amplitude of 0.5 mT. Experiments were carried out at 110 K using a liquid nitrogen cryostat.

EPR samples were prepared from the stock solution of peptide and/or ligand diluted down to 0.2 mM in H₂O. 0.9 equiv. of ⁶⁵Cu(II) was added from 25 mM ⁶⁵Cu(NO₃)₂ stock solution prepared in house from a copper foil (Eurisotop). Concentration was determined by formation of the Cu(BCS)₂³⁻ (BCS = bathocuproinedisulfonic acid, from Sigma-Aldrich) complex in the presence of an excess of ascorbate, using a molar extinction coefficient at 483 nm of 13 000 M⁻¹ cm⁻¹. Samples were frozen in a quartz tube after addition of 10% glycerol as a cryoprotectant and stored in liquid nitrogen until use.

UV-Visible spectroscopy

UV-Vis experiments were performed on an Agilent 8453 UV-Vis spectrometer at 25 °C.

Fluorescence spectroscopy

Fluorescence spectra were recorded by using a Fluostar Optima (BMG Labtech) connected to a personal computer.

Thioflavin T measurements

Thioflavin T (ThT), Aβ, Cu(II), Zn(II) and ligands at respective concentrations of 10 μM, 20 μM, 18 μM, 18 μM and 20 μM were mixed in 50 mM phosphate buffer, pH 7.1 and placed in a 384-well microplate. The time course of ThT fluorescence was then measured (excitation 440 nm; emission 490 nm, bandwidth for emission and excitation 10 nm).

Atomic force microscopy

Tapping-mode AFM imaging was performed in air using a Smart SPM-1000 microscope (AIST-NT, Novato, USA) equipped with a 100 μm scanner. Sample solutions (20 μM) were deposited on freshly cleaved mica and left for adsorption on the substrate for 10–20 min. They were then rinsed three times with deionized water to remove salts and loosely bound peptide and dried under compressed air before imaging. Commercial Si cantilevers (NanoWorld, Switzerland) with an elastic modulus of ~42 N m⁻¹ were used. All images were acquired as 512 × 512 pixel images at a typical scan rate of 1.0 kHz with a vertical tip oscillation frequency of 250–350 kHz. Representative images of samples were obtained by scanning at least 5 different locations on at least two different samples of the same chemical system.

Coumarin-3-carboxylic acid assay

3-CCA was used to detect HO[•]. HO[•] reacts with 3-CCA to form 7-hydroxy-coumarin-3-carboxylic acid (7-OH-CCA), which is fluorescent at 452 nm upon excitation at 395 nm. Under the conditions used in the present study the intensity of the fluorescence signal is proportional to the number of 7-OH-CCA molecules formed. Performing the experiment in phosphate

buffer is mandatory to detect formation of HO[•] since other buffers trap HO[•].

Ascorbate consumption assay

Ascorbate consumption was monitored by UV-Vis. Intensity of the Asc absorption band at λ = 265 nm (ε = 14 500 M⁻¹ cm⁻¹) was monitored as a function of time, with the background signal at λ = 800 nm subtracted.

Acknowledgements

The authors acknowledge Prof. E. Guillon, Drs S. Sayen and F. Collin, and C. Cheignon for their help in the recording of the XANES data; Dr O. Berthoumieu and M. Tasse for their help in acquiring the AFM pictures; L. Rechignat for the recording of the EPR data and Dr S. Noël for the L₂ synthesis. The authors acknowledge the European Synchrotron Radiation Facility for provision of beamtime and the FAME staff for their support (20151029). CH thanks the ERC aLzINK – Contract no. 638712 for financial support.

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