Electronic Supplementary Material (ESI) for Dalton Transactions.

Is ascorbate Dr Jekyll or Mr Hyde in the Cu(Aβ) mediated oxidative stress linked to Alzheimer's Disease?

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Supporting Information

Material and methods

Chemicals

Cu(II) used was from $CuSO_4.5(H_2O)$ and purchased from Sigma. Stock solution of Cu(II) was prepared in milliQ water (18.2 mΩ). Monobasic and dibasic phosphate buffers were bought from Sigma-Aldrich, dissolved in ultrapure water to reach a 0.1 M concentration mixed together to obtain a pH of 7.4. POPSO was bought from Sigma-Aldrich, dissolved in milliQ water to reach a 0.4 M concentration and adjusted to pH 9.0 with NaOH or H_2SO_4. Ascorbate solutions (20 mM and 0.2 M) were freshly prepared few minutes prior to each experimental set by dissolving sodium ascorbate (Aldrich) in milliQ water. A stock solution of coumarin-3-carboxylic acid (CCA) 1 mM (from Sigma) was prepared in phosphate buffer (0.1 M, pH 7.4). A stock solution of 7-hydroxycoumarin-3-carboxylic acid (7-OH-CCA) 1 mM (from Sigma) was prepared in milliQ water.

Peptide

Amyloid beta peptides were bought from GeneCust (Dudelange, Luxembourg), with purity grade > 95%. Stock solution of the $A\beta_{16}$ (sequence DAEFRHDSGYEVHHQK) and $A\beta_{28}$ (sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNK) peptides were prepared by dissolving the powder in milliQ water (resulting pH ~ 2). Peptide concentration was then determined by UV-visible absorption of Tyr10 considered as free tyrosine (at pH 2, $(\varepsilon_{276}-\varepsilon_{296})$ = 1410 $M^{-1}cm^{-1}$). Stock solution of $A\beta_{40}$ peptide (sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) was prepared by dissolving the powder in NaOH (50mM) and passing the solution through FPLC to obtain the monomeric fraction. The peptide concentration was then determined in NaOH (50mM) by UV-visible absorption of Tyr10, considered as free tyrosine ($(\varepsilon_{293}-\varepsilon_{360})$=2400 $M^{-1}cm^{-1}$).

Mass spectrometry

High Performance Liquid Chromatography / High Resolution Mass Spectrometry (HPLC/HRMS) analysis was performed on a LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Les Ulis, France) coupled to an Ultimate 3000 LC System (Dionex, Voisins-le-Bretonneux, France). The Orbitrap cell was operated in the full-scan mode at a resolution power of 60 000. Samples were washed three times with water prior analysis,by using Amicon 3 kDa centrifugal device (Millipore). Samples (10 µL) were then injected onto the column (Acclaim 120 C18, 50 × 3 mm, 3 µm, ThermoScientific), at room temperature. The gradient elution was carried out with formic acid 0.1% (mobile phase A) and acetonitrile/water (80/20 v/v) formic acid 0.1% (mobile phase B) at a flow-rate of 0.5 $mL.min^{-1}$. The mobile phase gradient was programmed with the following time course: 5% mobile phase B at 0 min, held 3 minutes, linear increase to 55% B at 8 min, linear increase to 100% of B at 9 min, held 2 min, linear decrease to 5% B at 12 min and held 3 min. The mass spectrometer was used as a detector, working in the full scan positive mode between 150 and 2 000 Da.

Fluorescence

Fluorescence experiments were performed on a multi-plate reader FLUOstar Optima 96-well plate reader system (BMG Labtech). Two automatic injectors were used to add ascorbate solutions into the wells during the experiments. Coumarin-3-carboxylic acid (CCA) was used to detect $HO^{\bullet}$. $HO^{\bullet}$ reacts with CCA to form 7-hydroxy-coumarin-3-carboxylic acid (7-OH-CCA), which is fluorescent at 450 nm upon excitation at 390 nm. Under the conditions used here, the intensity of the fluorescence signal is proportional to the number of 7-OH-CCA molecules formed, which in turn is proportional to the $HO^{\bullet}$ radicals trapped (Figure S1). Ascorbate is added to phosphate buffered (pH 7.4, 50 mM) solutions containing CCA (0.5 mM) and Cu^{II} (10/50/100 µM) with or without 1.2 equivalent of Aβ peptide ($A\beta_{16}$, $A\beta_{28}$ or $A\beta_{40}$). The final concentration of ascorbate in wells is between 0 and 5 mM. Two stock solutions of ascorbate (0,2 M and 20 mM) are used to cover the range of concentrations.

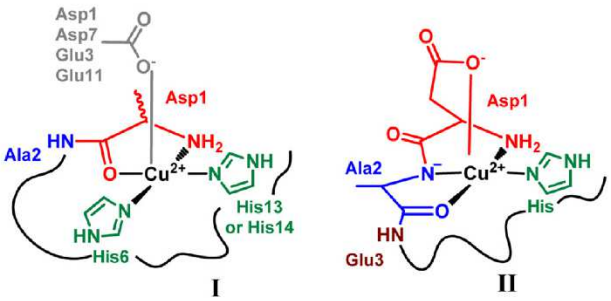


Figure S1: Schematic view of the proposed Cu(II) binding site in Aβ in component I (left) and II (right). pKa (I/II) = 7.8 [1]

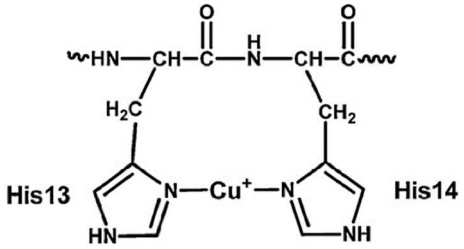
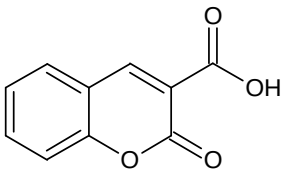
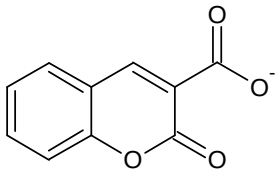


Figure S2: Schematic view of the proposed Cu(I) coordination site in Aβ.[1]

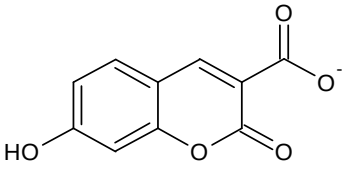
Fluorescent detection of HO• by CCA

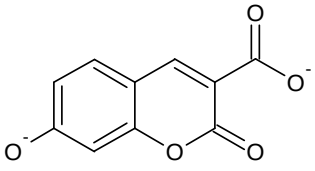
Fluorescence emission of 7-OH-CCA is very sensitive to pH as, the hydroxyl-group has a pKa of 7.5 (Figures S2 and S3), and only the deprotonated form emits at 450 nm after excitation at 390 nm. Since the experiment is performed at pH 7.4, i.e. very close to the pKa, the pH has to be strictly stable in order to have accurate results. Because the oxidation of ascorbate releases protons, it is not easy to keep the pH constant even with a high concentrated buffer, this issue being more important at higher ascorbate concentrations. Therefore, we established a new methodology to take into account this possible pH drift: (i) For the gradient measurement (Figure 2 in Full Text), we have verified that the pH drift is negligible even at high ascorbate concentration. This is due to the weak ascorbate consumption during the first minutes of the experiment. (ii) For the plateau measurement (Figure S5 and figure 3 in the Full Text), we have established a new protocol in which the pH was raised at the end of the reaction to pH 8.5, a region where 7-OH-CCA fluorescence is weakly sensitive to pH changes. Additionally, we have also verified that the rate of HO• trapped by CCA was independent of pH in the range of pH 7.0 to 7.4, where 7.0 is the minimal value of final pH obtained after experiment with high ascorbate concentration (Figure S4). Thus, the value of the measured fluorescence is proportional to the HO• trapped at pH 7.4.



Coumarin-3-carboxylic acid / 

Coumarin-3-carboxylate (pKa 3.3)



7-hydroxycoumarin-3-carboxylate / 

7-oxidocoumarin-3-carboxylate (pKa 7.5)

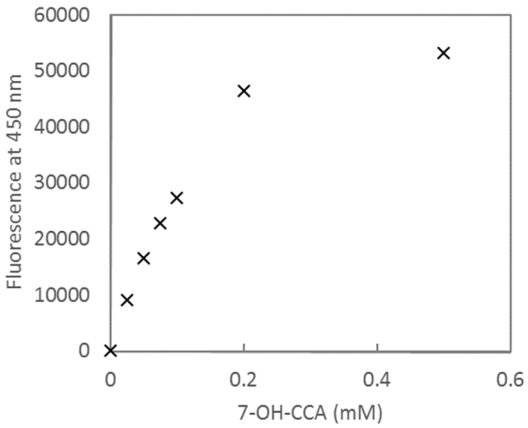


Figure S3: Fluorescence at 450 nm as a function of 7-OH-CCA concentration, after excitation at 390 nm. Phosphate-buffered solution (50 mM, pH 7.4) of 7-OH-CCA (concentration range from 0 to 0.5 mM).

Since the higher fluorescence intensity obtained in the present study (recorded under the very same conditions) is 14000, the fluorescence value is proportional to the 7-OH-CCA and the inner filter effect has not to be taken into account.

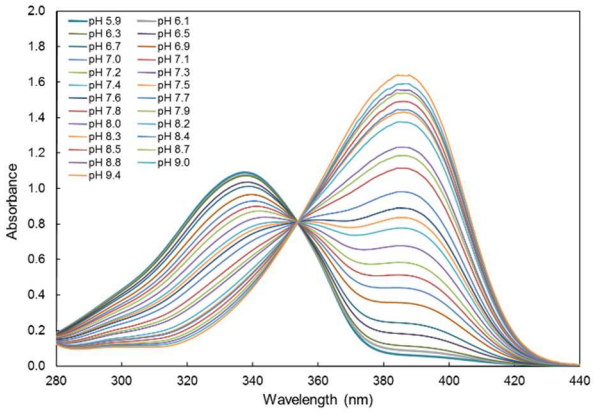


Figure S4: UV-Visible spectra of the 7-OH-CCA (50 µM) in a phosphate buffered solution (50 mM) with pH increased from 5.9 to 9.4 at 25°C.

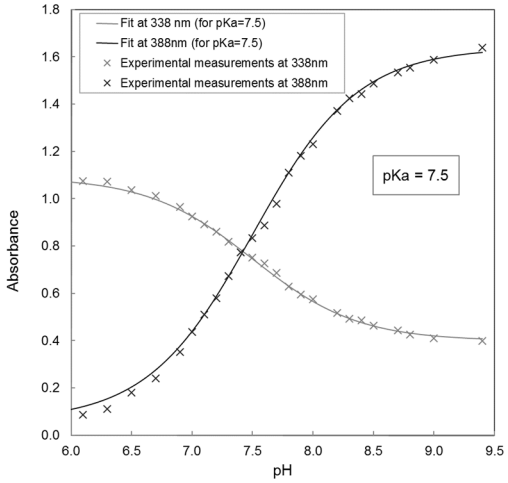


Figure S5: Determination of the pKa of the hydroxyl functional group of the 7-OH-CCA. Absorbance is plotted as a function of the pH of the acidic form absorbing at λ_{max} = 338 nm (grey crosses) and the basic form absorbing at λ_{max} = 388 nm (black crosses).

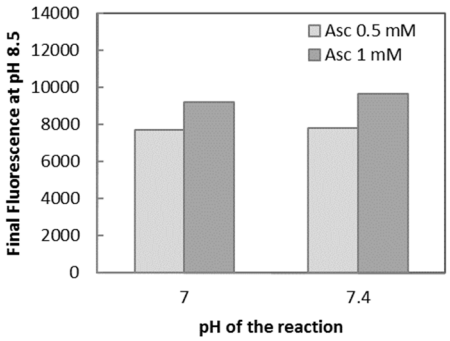


Figure S6: Impact of the pH on the formation of 7-OH-CCA during the metal-catalyzed production of the HO•. Reaction of Cu (50 µM), ascorbate (0.5 mM for grey panel and 1 mM for dark panel) in phosphate buffered solution (50 mM, pH 6.5, 7.0, 7.4, 8.0 or 8.5) containing CCA (0.5 mM). At the end of the reaction, the pH is ajusted to 8.5 with POPSO buffer (0.4 M, pH 9.0).

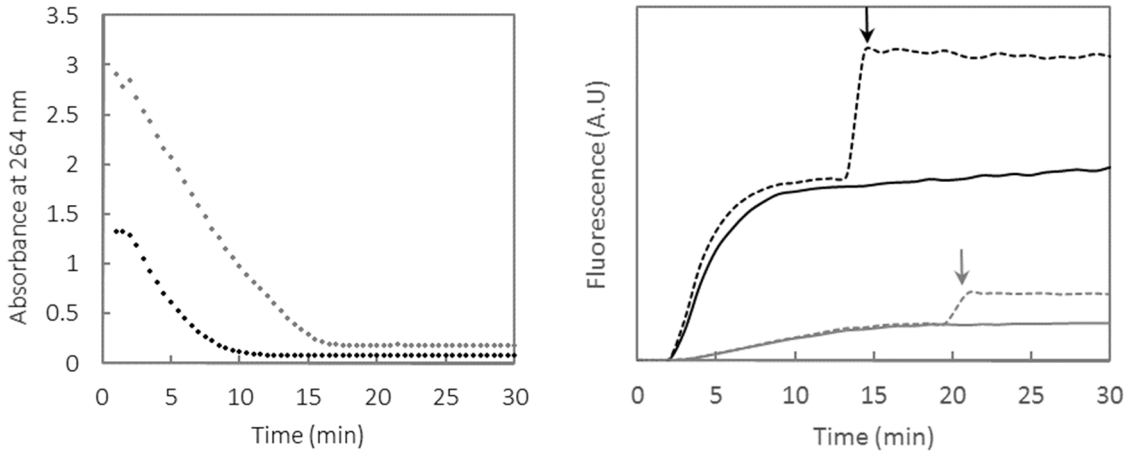


Figure S7: Reaction of Cu (50 µM, black curves) or Cu-$A\beta_{16}$ (50-60 µM, grey curves) with ascorbate (0.5 mM, in phosphate buffered solution, pH 7.4). Left: Ascorbate consumption followed using UV (absorption of ascorbate at λ_{max} = 264 nm) as a function of the time. Right: Fluorescence of the 7-OH-CCA produced by HO• trapping by CCA (0.5 mM) as a function of the time. When ascorbate is fully consumed (see left figure), POPSO buffer is added to increase the pH to 8.5. Arrow indicates the addition of POPSO on dotted curve.

LC-MS Experiments

Table S1: monoisotopic apparent masses (m/z) of mono- and multi-protonated ions of non-oxidized $A\beta_{28}$. m/z values in blue are used for $A\beta_{28}$ detection in HRMS.

Name	Sequence	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$	$[M+5H]^{5+}$
$A\beta_{28}$	DAEFRHDSGYEVHHQKLVFFAEDVGSNK	3261.5353	1631.27156	1087.85032	816.139694	653.11332

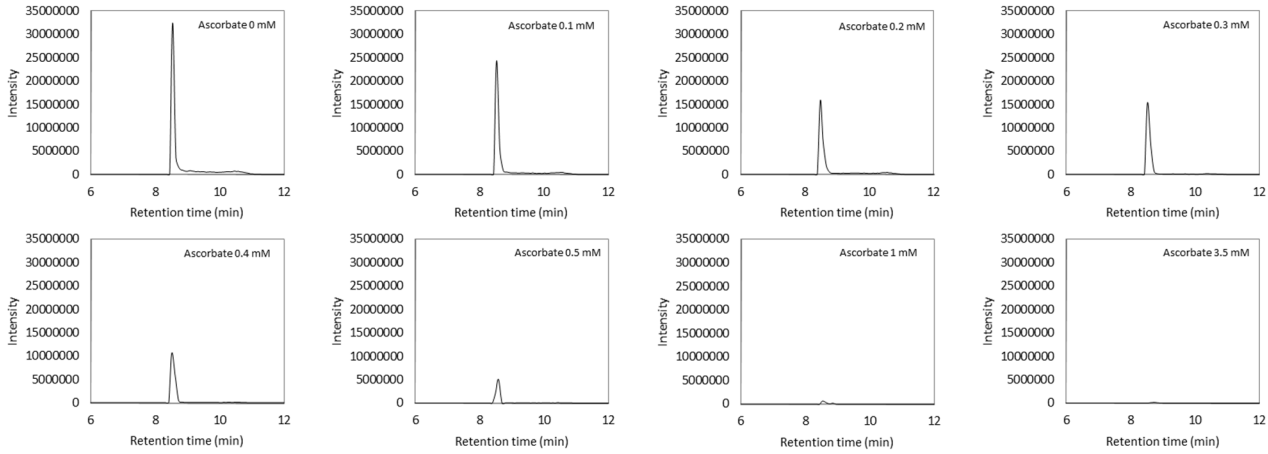


Figure S8: Trace chromatograms of the non-oxidized $A\beta_{28}$ peptide (60 µM) in the presence of Cu^{II} (50 µM) and ascorbate (0, 0.1, 0.2, 0.3, 0.4, 0.5 or 3.5 mM) at the end of the reaction. Mass tolerance set at 5 ppm; m/z ratios used for detection are specified in blue in table S1.

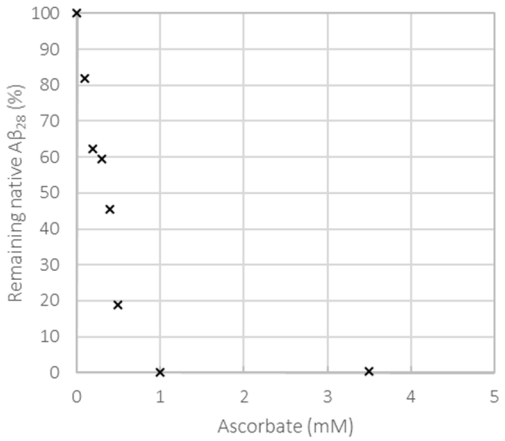


Figure S9: Remaining non-oxidized $A\beta_{28}$ at the end of the ROS production as a function of the ascorbate concentration. Phosphate-buffered solution (50 mM, pH 7.4) of $A\beta_{28}$ (60 µM), Cu^{II} (50 µM) and ascorbate (concentration range from 0 to 3.5 mM) analyzed in HPLC/HRMS. $A\beta_{28}$ peptide is detected with the monoisotopic masses (m/z) given in blue in table S1. Mass tolerance set at 5ppm. Trace chromatograms are given in Figure S8.

Since similar results are obtained whatever the length of the peptide (see Figure S10), the MS experiments were performed with $A\beta_{28}$ instead of $A\beta_{16}$ for technical reason.

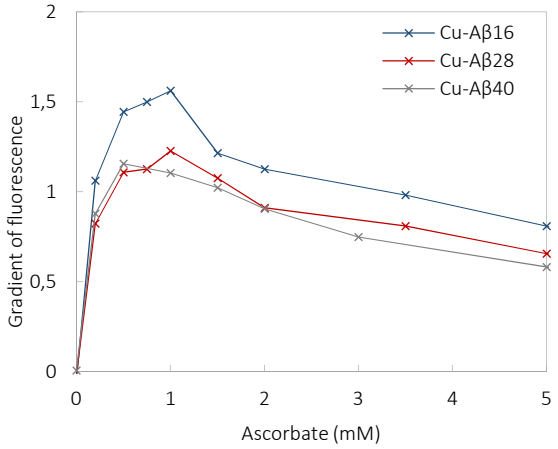


Figure S10: Initial rates of 7-OH-CCA fluorescence at 450 nm, reflecting the scavenging of $HO^{\bullet}$ by CCA. Phosphate-buffered solution (50 mM, pH 7.4) of CCA (0.5 mM), Cu (50 µM),), $A\beta_{16}$, $A\beta_{28}$ or $A\beta_{40}$ (60 µM) and ascorbate (concentration between 0 and 5 mM).

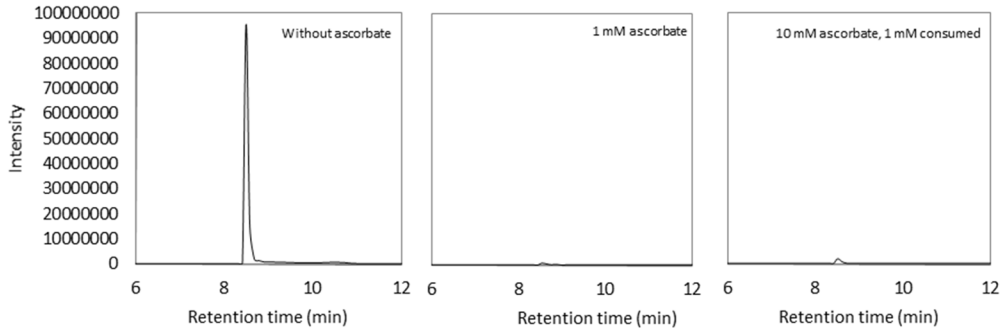


Figure S11: Trace chromatograms of the remaining non-oxidized $A\beta_{28}$ peptide (60 µM) after the addition of Cu^{II} (50 µM) and ascorbate (0 mM, 1 mM or 10 mM with only 1 mM consumed). Mass tolerance set at 5 ppm ; m/z ratios used for detection are specified in blue in table S1.

Table S2: Remaining non-oxidized $A\beta_{28}$ peptide (%) after the addition of Cu^{II} (50 µM) and ascorbate (0 mM, 1 mM or 10 mM with only 1 mM consumed). Percentages are calculated with the peak areas of the chromatograms traces (see Figure S11).

Starting ascorbate (mM)	Consumed ascorbate (mM)	Remaining $A\beta_{28}$ (%)
0	0	100%
1	1	1%
10	1	4%

1. Hureau, C., Coordination of redox active metal ions to the amyloid precursor protein and to amyloid-β peptides involved in Alzheimer disease. Part 1: An overview. *Coordination Chemistry Reviews* **2012,** *256* (19–20), 2164-2174.