The Necessity of Having a Tetradentate Ligand to Extract Copper(II) Ions from Amyloids

Michel Nguyen,[a] Lionel Rechignat,[a] Anne Robert,[a][b] and Bernard Meunier[a][b]

The accumulation of redox-active metal ions, in particular copper, in amyloid plaques is considered to be the cause of the intensive oxidation damage to the brain of patients with Alzheimer’s disease (AD). Drug candidates based on a bis(8-aminoquinoline) tetradentate ligand are able to efficiently extract Cu²⁺ from copper-loaded amyloids (Cu–Aβ). Contrarily, in the presence of a bidentate hydroxyquinoline, such as clioquinol, the copper is not released from Aβ, but remains sequestered within a Aβ–Cu–clioquinol ternary complex that has been characterized by mass spectrometry. Facile extraction of copper(II) at a low amyloid/ligand ratio is essential for the re-introduction of copper in regular metal circulation in the brain. As, upon reduction, the Cu⁺ is easily released from the bis(8-aminoquinoline) ligand unable to accommodate Cu⁺, it should be taken by proteins with an affinity for copper. So, the tetradentate bis(8-aminoquinoline) described here might act as a regulator of copper homeostasis.

The pathology of Alzheimer’s disease (AD) is related to the abnormal deposition of two proteins, amyloid proteins (Aβ) and hyperphosphorylated tau protein, as described by Alzheimer a century ago.[1] The rupture of the homeostasis of two redox-active metal ions, namely copper and iron, in AD brain, and their accumulation in senile plaques has been largely documented.[2,3]

The strong binding of copper and iron ions with amyloids, their role in the excessive reticulation of Aβ peptides[4, 5] and in the intense oxidative damage evidenced in AD brain[6-8] have been documented. The catalytic formation of reactive oxygen species (ROS) generated by redox-metal-loaded amyloids, and responsible for Aβ toxicity, has been reported.[9,10]

In order to decrease the toxicity of Cu–Aβ due to the easy reduction of Cu²⁺ to Cu⁺ under physiological conditions, we designed copper-chelating agents able 1) to retrieve Cu ions from Cu–Aβ, and 2) to transfer these copper ions to regular carrier proteins for regular copper circulation in the brain. Here, we report that bis(8-aminoquinoline) ligands,[11] tetradentate ligands able to chelate Cu⁺ with a ligand-metal stoichiometry of 1:1,[12] are able to extract copper(II) at low amyloid/ligand ratios. For comparison, clioquinol (CQ), a bidentate 8-hydroxyquinoline formerly used as antipROTOzoal drug and recently developed as a metal regulator for the treatment of AD,[13] is unable to extract copper ions from Cu–Aβ, but forms a ternary complex Aβ–Cu–CQ. For economic and scientific reasons, we used Aβ1–28 (Figure 1) and Aβ1–16 instead of Aβ1–42. These two short peptides contain the Cu⁺ coordination site (Asp1, His6, His13,14) of the N terminus of Aβ peptides, considered to be independent of amyloid length and responsible, at least in part, for ROS production in AD pathology.[14-16] In addition, these truncated peptides, behaving as monomers, are likely to be relevant models of longer amyloids.

The transfer of copper from Cu–Aβ amyloids to bis(8-aminoquinoline) ligand 1 (Figure 1) was monitored by UV-visible spectrometry, electron spin resonance (ESR) spectroscopy, and mass spectrometry to characterize copper complexes Cu–Aβ and Cu–1.

![Bis(8- amino)quinoline](1)

![Clioquinol (CQ)]

Figure 1. Structures of bis(8-aminoquinoline) ligand 1, clioquinol (CQ), and Aβ1–28.

First, we metalated Aβ1–28 with 1 mol equiv of CuCl₂ at room temperature. As previously reported, metalation was instantaneous and proceeded to completion, as evidenced by the decrease by 50% of the tyrosine-10 fluorescence.[14] The ESR spectrum confirmed the chelation of copper(II) by Aβ (see below). Bis(8-aminoquinoline) ligand 1 was then added (1 mol equiv with respect to Cu–Aβ).

The UV-visible spectrum of the resulting mixture, Cu–Aβ/ligand 1, was superimposable on the spectrum of the complex...
Cu–1 (λ\text{max} = 277, 329, 354, 367 nm), indicating that the copper ion was completely extracted from Cu–Aβ and transferred to ligand 1 (Figure 2a).

By comparison, a similar experiment was carried out with CQ instead of ligand 1. When 2 mol equiv of CQ were added to Cu–Aβ,28, the resulting UV-visible spectrum exhibited an absorbance at 438 nm (Figure 2b). This spectrum, significantly different from that of complex Cu(CQ)2 (456 nm, Figure 2b), was assigned to a ternary Aβ1–28/CQ complex. A similar ternary complex, but with an 8-hydroxyquinoline analogue of CQ, has been previously reported on the basis of ESR experiments. These data indicated that 2 mol equiv of CQ failed to completely extract Cu\textsuperscript{2+} from Cu–Aβ. It should be noted that in a different solvent mixture, namely acetonitrile/HEPES buffer (10:90 v/v), bands were broader, and it was not possible to unambiguously distinguish the spectra of Cu(CQ)\textsubscript{2} (λ\text{max} = 450 ± 2 nm) from a putative Aβ/Cu–CQ complex (λ\text{max} = 446 ± 2 nm).[12]

The fast migration of Cu\textsuperscript{2+} from Aβ to ligand 1 was confirmed by ESR spectroscopy. The analysis solvent was HEPES buffer (100 mM, pH 7.4) containing 1–3 vol% of DMSO. The spectrum of Cu–Aβ1–16 exhibited an \( A_{ij} \) value of 176 ± 3 G with a \( g_{ij} \) value of 2.265 ± 0.004 (Figure 3a). Upon addition of ligand 1 (1 mol equiv; Figure 3c), the spectrum exhibited an \( A_{ij} \) value at 204 G with a \( g_{ij} \) value of 2.196 ± 0.002, significantly different from the values for Cu–Aβ1–16. Furthermore, the spectrum was superimposable on the spectrum of Cu–1 in the absence of Aβ (Figure 3d). In addition, the spectrum of Cu–Aβ1–16 in the presence of 0.5 mol equiv of 1 could be assigned to an equimolecular mixture of Cu–Aβ1–16 and Cu–1 (Figure 3b). Given these results, it has not been possible to evidence in the process of extracting Cu\textsuperscript{2+} from Aβ1–28 by 1 any putative copper complex containing both Aβ and 1 as ligands. The hyperfine coupling constants \( A \) and \( g \) factors are summarized in Table 1.

In addition, for Cu–1, and despite quite broad lines due to the presence of copper ions in natural abundances, the second-derivative spectrum centered on the \( g \) factor of Cu–1 exhibited nine lines with relative intensities 1/2/3/2/2/2/1. This pattern can be assigned to the overlap of two

![Figure 2](https://example.com/figure2.png)

**Figure 2.** a) Extraction of Cu\textsuperscript{2+} from Aβ1–28 upon addition of 1, evidenced by UV-visible spectroscopy. Spectrum of Cu–Aβ1–28 + 1 (Cu–Aβ1–28/CQ mol ratio = 1:1; −−−−) compared with those of Cu–1 (−−−−) and 1 (−−−−−−−−−); b) Non-extraction of Cu\textsuperscript{2+} from Aβ1–28 upon addition of clioquinol (CQ), evidenced by UV-visible spectroscopy. Spectrum of Cu–Aβ1–28 + CQ (Cu–Aβ1–28/CQ mol ratio = 1:2; −−−−), Aβ1–28 and Cu–Aβ1–28 do not significantly absorb in this wavelength range. For experimental details, see the Experimental Section.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Electron spin resonance (ESR) spectra of a) Cu\textsuperscript{2+}–Aβ1–16, b) Cu–Aβ1–16/CQ (1:0.5 mol ratio), c) Cu–Aβ1–16/CQ (1:1 mol ratio), d) Cu–1, in HEPES buffer containing 1–3 vol% DMSO.

<table>
<thead>
<tr>
<th>( A_{ij} ) [G]</th>
<th>( g_{ij} )</th>
<th>( g_z )</th>
<th>( A_n ) [G]</th>
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<tr>
<td>Cu–Aβ1–16</td>
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<td>2.053</td>
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<tr>
<td>Cu–Aβ1–16/CQ</td>
<td>204</td>
<td>2.196</td>
<td>2.025</td>
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<tr>
<td>Aβ–Cu–CQ</td>
<td>193</td>
<td>2.225</td>
<td>2.069</td>
</tr>
</tbody>
</table>

[a] In HEPES buffer containing DMSO (1–3 v%). [b] In DMSO/HEPES buffer (90:10 v/v). [c] not determined (n.d.)
Figure 4. Second-derivative electron spin resonance (ESR) spectrum of CuII–1. 

Figure 5. Electron spin resonance (ESR) spectra of a) CuII–Aβ1–16, b) CuII–Aβ1–16, w/CQ (1:1 mol ratio), c) CuII–Aβ1–16, w/CQ (1:2 mol ratio), d) CuII(CQ)2 in DMSO, containing 10 vol% of HEPES buffer.

quintuplets with intensities 1/2/3/2/1 due to the complexation of two aniline-type and two quinoline-type nitrogen atoms. This indicates that the complexation of copper by a bis(8-aminonquinoline) evidenced in the solid state by X-ray crystallography was retained in solution. The super hyperfine pattern of Cu–1 was also detectable in the mixture containing Aβ1–16/CuII/1 in a 1:1:1 ratio (Figure 3c).

The mixture resulting from addition of CQ in pre-formed Cu–Aβ was analyzed by ESR spectroscopy for comparison. Due to the poor aqueous solubility of CQ and its copper complex Cu(CQ)2, analyses were performed in DMSO containing 10 vol% of HEPES buffer, pH 7.4.

Note that the spectrum of CuII–Aβ1–16, under these conditions (Figure 5a) was significantly different than that recorded in HEPES containing only 1–3 vol% of DMSO (Figure 3a), where Aβ1 was 114 and 176 G, respectively, and g|| was 2.408 and 2.265, respectively (Table 1), indicating that is likely to be involved in the coordination sphere of CuII. When CQ was added to CuII–Aβ1–16 (Cu–Aβ/CQ = 1:1 and Cu–Aβ/CQ = 1:2; Figure 5b,c, respectively), the signal of CuII–Aβ1–16 disappeared and a series of resonances different from that of CuII(CQ)2 appeared with an A|| value of 193 G and a g|| value of 2.225, along with that of CuII(CQ)2 (Figure 5d). This feature suggests that both CQ and Aβ1–16 were acting as ligands of CuII.

The ternary complex Aβ1–16–Cu–CQ was further characterized by mass spectrometry (MS). The Cu–Aβ1–16 complex was first prepared; 1 mol equiv of CQ was then added, and the mixture was immediately analyzed by MS using positive-mode electrospray ionization (ESI+). Along with the peaks corresponding to Aβ1–16 (m/z = 9785, 6526.2, 489.7, and 392.0 amu, for z = 2, 3, 4, and 5, respectively), a series of multicharged peaks was detected at m/z 1161.9, 774.9, and 581.4 amu with z = 2, 3, and 4, respectively (Figure 6a). This pattern can be assigned to a ternary complex Aβ1–16–Cu–CQ with molecular formula C69H112ClCuN9O26. The isotopic patterns were consistent with the theoretical profiles (Figure 6b). The complex Cu–Aβ1 was also detected at m/z 1008.9, 672.9, 505.0, and 404.2 (z = 2, 3, 4, and 5, respectively), indicating that a significant amount of Cu–Aβ1 was not affected by the presence of CQ (ca. 20% with respect to free Aβ1; for the full-scale spectrum, see Figure S1 in the Supporting Information).

Under the same conditions, in a mixture containing Cu–Aβ1 and ligand 1, signals of a putative complex Aβ–Cu–1 were not detected (Figure S2 in the Supporting Information). The major detected compounds were free Aβ1 and CuII–1 (m/z = 421.1 [M–H]+ and m/z = 211.1 [M]+). Only a tiny amount of Cu–Aβ1 was detected (<4% with respect to free Aβ1), indicating a major demetalation of Aβ1.

This result is consistent with the affinity constants of Aβ1 and the different ligands for CuII. In fact, the apparent log aff value of Aβ1 for CuII was reported to be in the range of 10–11,[14,15] very closed to that of CQ (log Kaff = 10).[18] As clearly evidenced in this report, a stable ternary complex Aβ–Cu–CQ was observed. Contrarily, the much higher affinity of ligand 1 for CuII (log Kaff = 16.5)[12] allows to obtain an efficient extraction of copper from Aβ1. It should be noted that ternary complexes involving Aβ1, ZnII and CQ,[19] or Aβ1, FeO and another metal chelating agent,[10] have been reported.

In conclusion, we have demonstrated that a tetradentate ligand is much more suitable for the extraction of copper(II) from copper-loaded amyloids than a simple bidentate ligand such as CQ. An easy extraction of copper(II) at low amyloid/ligand ratio is essential for AD metal regulators in order to facilitate the re-introduction in copper circulation in the brain.

Experimental Section

Aβ1 peptides were purchased from Bachem, Switzerland. The content of each peptide flask was dissolved by addition of HEPES buffer 100 mM, pH 7.4 (Aβ1, 10 μg) or ultrapure Milli-Q water (Aβ1, 10 μg). The concentration of Aβ1 was then measured by UV-visible spectroscopy (ε278nm (Ytr10) = 1410 mμmol·cm−1).14

UV-visible spectra were recorded on a Biochrom Libra S50 or a Spectord 205 spectrophotometer (Analytik Jena, Germany). Fluorescence spectra were recorded on a FLSP920 spectrometer (Edinburgh Instruments Ltd, UK), with bandwidth for excitation and
emission = 2 nm. The Cu–A\textsubscript{b} complex was first prepared by mixing equimolar amounts of A\textsubscript{b}1–28 and CuCl\textsubscript{2} in HEPES buffer 50 mM, pH 7.4. The metalation of A\textsubscript{b} was monitored by the decrease of fluorescence (see Ref. [14]). A solution of ligand 1 or CQ in DMSO was then added (1 or 2 mol equiv, respectively), and the reaction was monitored by UV-visible spectroscopy. Final concentrations were [A\textsubscript{b}1–28] = [Cu\textsubscript{2}\textsuperscript{2+}] = [1] = 20 mM, [CQ] = 40 mM; DMSO/HEPES buffer = 5:95 v/v. The 50 % decrease of fluorescence of A\textsubscript{b} upon metalation by copper was confirmed in buffered mixture containing up to 10 vol % of an organic solvent, namely CH\textsubscript{3}CN (Ref. [12]), or DMSO (present report, data not shown).

X-Band (9.525 GHz) ESR spectra were recorded in quartz tubes at 120 K, using a Bruker Elexsys-II E500 spectrometer. For experiments with ligand 1, the solvent was HEPES buffer 100 mM, pH 7.4, containing 1–3 vol % of DMSO. [A\textsubscript{b}1–16] = 185 µM; [A\textsubscript{b}1–16/Cu molar ratio] = 1:1 (Figure 3 a), [A\textsubscript{b}1–16/Cu/1 = 1:1:0.5] (Figure 3 b), [A\textsubscript{b}1–16/Cu/1 = 1:1:1] (Figure 3 c), Cu/1 = 1:1 (Figure 3 d). The addition of 1–6 vol % of DMSO in HEPES did not induce modification of the spectrum of Cu–A\textsubscript{b}1–16 (data not shown). For experiments with CQ, the solvent was DMSO/HEPES buffer 100 mM, pH 7.4, 90:10 v/v. [A\textsubscript{b}1–16] = 280 µM; [A\textsubscript{b}1–16/Cu molar ratio] = 1:1 (Figure 5 a), [A\textsubscript{b}1–16/Cu/CQ = 1:1:1] (Figure 5 b), [A\textsubscript{b}1–16/Cu/CQ = 1:1:2] (Figure 5 c), Cu/CQ = 1:2 (Figure 5 d).

ESI-MS analyses were performed on a Waters Xevo-G2QTOF mass spectrometer. The sample solutions were injected (7.5 µL) using a mobile phase CH\textsubscript{3}OH/H\textsubscript{2}O (90:10 v/v), flow rate = 0.15 mL min\textsuperscript{-1}. The cone voltage was 15 V, and spectra were acquired in the positive ion mode, in the m/z range 100–2500. The mixture of A\textsubscript{b}1–16/CuCl\textsubscript{2}/CQ (1:1:1 mol ratio) was prepared in ultrapure Milli-Q water (pH 5.8)/MeOH (1:1 v/v). Final concentration was 100 µM, injected volume was 7.5 µL. The series of multicharged patterns at m/z = 1161.9, 774.9, and 581.4 was not detected in the absence of Cu\textsuperscript{2+}.

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