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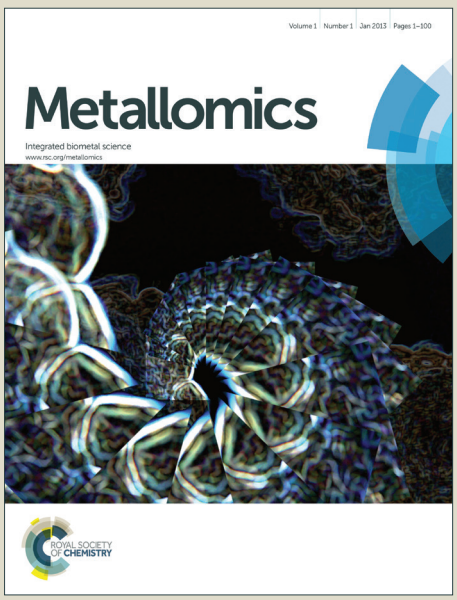

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COMMUNICATION

Copper(I) targeting in the Alzheimer's disease context: a first example using the biocompatible PTA ligand

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Copper(I) coordinating ligands in the Alzheimer's disease context have remained unexplored, despite the biological relevance of this redox state of the copper ion. Here, we show that the PTA ligand can remove copper from Aβ, prevent reactive oxygen species production and oligomers formation, two deleterious events in the disease's etiology.

It is estimated that one out of twenty people over 65 years old suffers from Alzheimer's disease (AD). In the early stages the main symptom is memory loss but within the time, AD patients lose other intellectual abilities, thus interfering with their daily life. As life expectancy has been generally growing in recent years, and keeps going up, AD is going to be an issue of paramount importance. Up to now there is no cure for AD, the only medications approved by the U.S. Food and Drug Administration are indicated to palliate the symptoms.

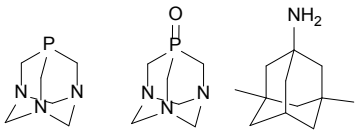
At a physiological level, two noteworthy hallmarks have been observed in brains of AD patients:[1] extracellular amyloid plaques containing high amounts of the amyloid-β (Aβ peptide) under aggregated forms and of metal ions (mainly copper and zinc),[2] and neurofibrillary intracellular tangles. Soluble monomeric Aβ peptide is found in healthy brains and because amyloid plaques are found in AD patients, it is thought that formation of the plaques is a key process for the etiology of AD, known as the amyloid cascade.[3] According to this hypothesis, aggregation of Aβ has been proposed to be a key and early event in the AD progression inducing further events of the disease processes, including formation of neurofibrillary tau tangles, local inflammatory response, etc. leading to the death of the neuronal cells and finally to dementia. Despite senile plaques are very important in AD, it has been found that soluble oligomers of Aβ (low molecular weight aggregates) would be even more toxic.[4, 5]

In addition, the brain is an organ rich in metal ions, and some of them (Zn and Cu) can be found in high levels in the hippocampus, the region of the brain related to memory.[6] They can bind to the Aβ peptide, impact its aggregation and also the production of Reactive Oxygen Species (ROS),[2] a second crucial element in AD.[7] In this context, Cu due to its redox ability is the target of choice for therapeutic purpose based on metal ions removal by chelators.[8]

As a consequence, an intensive field of research has recently developed following this approach. However, it is worth noting that molecules described until now in the literature are all competent to sequester the +II redox state of Cu[8] and sometimes to redox silence it[7] but that there is no data reported regarding ligands specific for the Cu^{I}. However, brain is a reducing environment and the extracellular ascorbate concentration can reach up to 300μM.[9] In addition, during production of ROS, the Cu ion cycles between the +I and +II redox states. It has also been shown that the biological Cu(I) chelator MT-3 (metallothionein-3) was more efficient in protecting cells from Cu-Aβ toxicity compared to HSA (Human Serum Albumine), a Cu(II) chelator.[10, 11] That's why the +I state of Cu is as much biologically relevant as the +II state.

The present study thus aims at evidencing that the use of Cu(I) ligands could be a prospective approach in the design of new therapeutic agents against AD. In this context, the ability of the phosphane 1,3,5-triaza-7-phosphadaamante (PTA, Scheme 1) to reduce Cu(II) and stabilize Cu(I) as a tetrahedral coordinated compound[12] has been exploited. PTA presents some advantages as its small cone angle, its resistance to oxidation compared to other phosphines, its solubility in water, its biocompatibility and low intrinsic toxicity.[13] Another important characteristic is that there are several positions through which the molecule can be modified by alkylation or arylation in the nitrogen atoms[14] among other examples. PTA and PTA derivatives coordination compounds have thus been studied for biomedical applications. In addition to the popular RAPTA complex, an organometallic species where a Ru(II) centre is bound to one PTA ligand, which has shown a promising activity against cancer cells,[15] Cu(I) - PTA complexes has also been developed.[13, 16]

In this communication, the (i) removal of Cu (Cu(I) and Cu(II)) from Aβ by PTA ligand and associated (ii) delay of ROS production and (iii) inhibition of oligomeric Cu-Aβ species formation are reported



Scheme 1. From left to right: PTA and PTA-oxide (O=PTA) molecules and memantine.

As a starting point, Cu removal from the Aβ[§] peptide using the PTA ligands was investigated. Several techniques were used. The most appropriate one is XANES (X-ray Absorption Near Edge Structure) spectroscopy since it is possible to follow the entire reaction pictured in Scheme 2. Indeed, both Cu(II) and Cu(I) are active in XANES. Figure 1 thus shows the evolution of the signature of the Cu^{II}(Aβ) species in presence of increasing equivalent of PTA ligands. In presence of 4.5 equiv. of PTA, the Cu^{II}(Aβ) spectrum (a) is replaced by the fingerprint of the $[Cu^{I}(PTA)_4]^+$ complex (spectrum c). 0.5 equiv. of PTA are necessary for Cu(II) reduction and 4 for the Cu(I) capture from Aβ. Increasing the number of PTA equivalents helps to accelerate the completion of the process (see note ⊥).

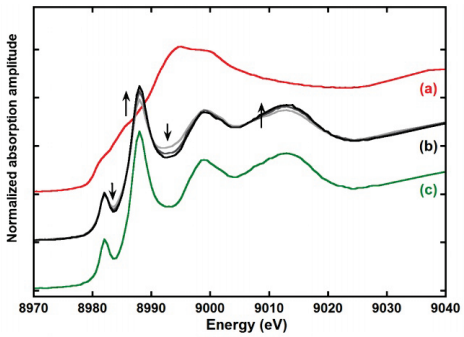
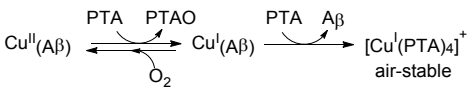


Figure 1. XANES spectra of a mixture of [Aβ16] = 1 mM and [Cu^{II}] = 0.9 mM (spectrum (a), red) and [PTA] from 4 to 6mM after approx. 10 min of incubation time (spectra (b) from grey to black) and of [PTA] = 5 mM and [Cu^{II}] = 0.9 mM leading to the *in situ* formation of $[Cu^{I}(PTA)_4]^+$ after approx. 10 min of incubation time (spectrum (c), green). Hepes buffer 50 mM pH 7.4, *T* = 20 K.



Scheme 2. Two-step process from Cu^{II}(Aβ) to $[Cu^{I}(PTA)_4]^+$. Stoichiometry of the reaction is not given for the sake of clarity. [Cu] in the mM range.

To ascertain the two-step process, we also recorded (i) the UV-Vis and EPR signatures of Cu^{II}(Aβ) after addition of 6 equiv. of PTA that evidence the Cu(II) reduction (Figures S1-S2) and (ii) the NMR spectra of a mixture of Cu^{I}(Aβ) and 6 equiv. of PTA that shows the transfer of Cu(I) to the PTA ligands (Figure S3). Note that reduction of Cu(II) by PTA ligands leading to O=PTA is evidenced by the formation of the characteristic NMR peak in $^{31}P\{^1H\}$-NMR (Figure S4), in line with similar oxydo-reduction process observed with other metallic ions.[17] However, in the present case, in contrast to what has been reported for Co(II),[18] Zn(II), Cd(II) and Hg(II)[19] complexes, no binding of the O=PTA to Cu(I) has been detected. Note also that quantitative formation of $[Cu^{I}(PTA)_4]^+$ complex observed here by spectroscopy is in agreement with the respective affinity constants of Aβ and PTA ligands for Cu(I).[20-22]

Then, the impact of Cu removal from Aβ and sequestration as the air-stable $[Cu^{I}(PTA)_4]^+$ complex on ROS production was investigated by established methods.[23] Briefly, the ROS production can be seen as the reduction of dioxygen by ascorbate catalysed by the $Cu^{I/II}$(Aβ) complex leading to $O_2^{\bullet-}$, H_2O_2 and HO°. Thus, either ascorbate consumption can be followed by UV-Vis at 265 nm or HO° formation can be monitored by the detection of the fluorescent 7-OH-CCA (7-hydroxy-coumarin-3-carboxylic acid) dye formed by reaction of HO° and the CCA (Coumarin-3-carboxylic acid) molecule (see experimental part in the SI for details).[7]
In Figure 2, formation of the fluorescent 7-OH-CCA dye is monitored as a function of time and of PTA equivalents with (panel B) and without Aβ (panel A). In absence of the Aβ peptide, impact of PTA is double: (i) it diminishes the number of 7-OH-CCA formed, since the values of the fluorescence plateau decrease as the number of PTA added is increased. This indicates that PTA could be oxidized (in competition with CCA and ascorbate) by HO°; (ii) it slows down the production of 7-OH-CCA, with a lag phase that increases with the number of PTA equivalents. This means that oxidation of PTA induces the Cu release and subsequent HO° production with a rate similar to what is observed in absence of PTA and that PTA in excess delays the Cu release. Two mechanisms, an direct one and a indirect one, can explain the oxidation of PTA: (ii) PTA reduces Cu(II) directly; (ii) $[Cu^{I}(PTA)_3]^+$, the main species present at low concentration is in equilibrium with redox-active $[Cu^{I}(PTA)_{0\text{-}2}]^+$ species (see Supporting information for details). In presence of ascorbate, the latter species produce ROS catalytically, leading to the predominant oxidation of PTA, in line with the reactions plotted in Scheme 3.
In presence of Aβ, the same trend is observed except that the slope of the lag phase is not as flat as in absence of Aβ. This is explained by the presence of significant amount of Cu(I) bound to Aβ (See supporting information for the calculations details) leading to a higher ROS production. For the same reason, the lag phases are shorter than in absence of Aβ. Note also that the level of 7-OH-CCA formed at the plateau is lower than in absence of Aβ. This is due to the oxidation of the Aβ itself (i.e. HO° attacks Aβ instead of CCA).

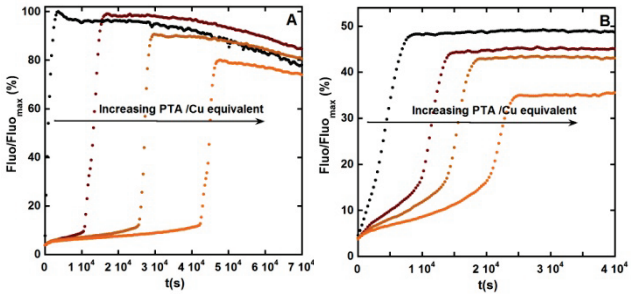
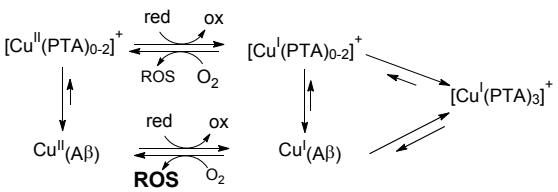


Figure 2. 7-OH-Fluorescence spectra as a function of time of unbound Cu (panel A) and Cu(Aβ) (panel B) in presence of increasing equivalent of PTA (no PTA added, black dots), 4 equiv. of PTA (brown dots), 5 equiv. of PTA (light brown dots) and 6 equiv. of PTA (orange dots). [Cu^{II}] = 10μM, [Aβ16] = 12 μM, [PTA] = 0, 42, 52, 63 μM, [CCA] = 500 μM, [ascorbate] = 1mM, phosphate buffer, 50mM, pH 7.4, T= 25°C.



Scheme 3. Catalytic production of ROS in presence of Cu and PTA or Cu plus Aβ plus PTA. red = reductant, i.e. ascorbate or PTA. The ROS formed can attack PTA, Aβ and CCA. PTA and Aβ ligands are not mentioned in the equation for matter of clarity. [Cu] in the 10 μM range.

Thus, PTA is highly efficient in stopping, at least for a period of time, ROS formation. This positive impact is observed regardless of the time of PTA addition during the ROS production process (Figure S5 for HO° formation and S6 for ascorbate consumption experiments). Hence, the PTA ligand is able to redox silence both the Cu^{I}(Aβ) and Cu^{II}(Aβ) species, which is a very important property if further therapeutic purpose are intended.

Finally, PTA propensity to modulate Aβ aggregation was determined by the classical ThT (Thioflavine-T) assay (Figure 3, left) and the nature of the aggregates formed (oligomers or fibrils) was determined by AFM (Figure 3, right).[24, 25] Under our working conditions, Cu(II)-modulated aggregation of Aβ leads to the formation of oligomeric and small protofibrils species (considered as the most toxic species in the aggregation process from the monomeric Aβ to the fibrils found in the senile plaques) exhibiting

very low ThT fluorescence and characteristic AFM pictures (Figure 3, left, (b) and right, bottom and Figure S7). In contrast, the Aβ-only peptide follows the classical aggregation pathway characterized by the sigmoid-shape curve of the ThT fluorescence (Figure 3, left, a)[24, 25] and forms amyloid-type fibrils (Figure 3, right, top and Figure S7). Addition of 20 equiv. of PTA into a solution of Cu^{II}(Aβ) results in the detection of a ThT fluorescence curve very similar to the one observed for aggregation of the apo-Aβ and to AFM picture that resembles those of the apo-fibrils (Figure S7). This indicates that similar amyloids are formed. When less PTA equivalents are added, the ThT curves and AFM pictures resemble to those of the Cu^{II}(Aβ) sample (Figure 3, dotted red line and Figure S7). This can be explained by reoxidation of the Cu(I) ion that happens when there is not enough reductant in the medium (Scheme 2). Hence, here the excess of PTA is used to keep the PTA:Cu(I) ratio above 4 despite the loss of PTA due to oxidation. In summary, the PTA ligand is able to extract the Cu from Aβ and preclude the formation of the oligomeric species.

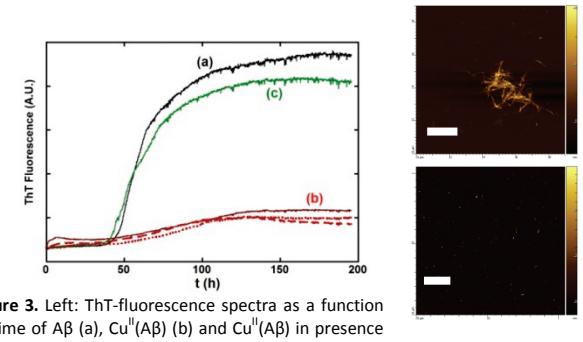


Figure 3. Left: ThT-fluorescence spectra as a function of time of Aβ (a), Cu^{II}(Aβ) (b) and Cu^{II}(Aβ) in presence of 20 equiv. of PTA (c). Dotted red curves are obtained in presence of 5 and 10 equiv. of PTA. [Cu^{II}] = 18 μM, [Aβ40] = 20 μM, [ThT] = 7 μM, phosphate buffer, 50mM, pH 7.0, T= 37°C. Right: AFM pictures corresponding to curve (a) (top) and (b) (bottom). Samples were withdrawn at t = 180 h. White scale corresponds to 2 nm.

Conclusions

In the present communication, we report the first Cu(I) ligand able to retrieve both Cu(I) and Cu(II) from the Aβ peptide, to redox silence them, thus retarding the formation of the toxic ROS species and finally to induce the formation of apo-type fibrils instead of toxic Cu(Aβ) oligomers. To the best of our knowledge this is the very first example of such a Cu(I) targeting ligand in a field where all the current researches are focusing on Cu(II) as a target despites both redox states of Cu are biologically relevant. Although, PTA can hardly be used as such against AD, the present study proves that it is worth considering the Cu(I) targeting approach. In addition, the versatility of the PTA ligand could be further used to increase its Cu(I) affinity thus its resistance to ROS production and its resistance to oxidation, so that PTA can reach its biological target without priori oxidation. Sophistications can also includes functionalization with Aβ recognition moiety in a strategy similar to the one developed for Cu(II) chelators.[8, 25] It will also be interesting to make some modifications to increase its structural similarity with the memantine scaffold (Scheme 1), a drug used to palliate some of the effects of AD and thus to propose a new kind of bi-functional therapeutic tool.

Notes and references

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[Supporting information include UV-Vis and EPR monitoring of Cu^{II}(Aβ) reduction by PTA, NMR spectra of Cu(I) exchange between (Aβ) and PTA, of O=PTA formation, HO° production and ascorbate consumption studies, details of speciation calculations, large AFM pictures of aggregation study and materials and methods description.]

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§ Note that the Aβ peptide used in the coordination and ROS production experiments are the N-terminally part encompassing the first 16 amino-acid residues (one code letter sequence: DAEFRHDSGYEVHHQK) known to well reproduce the Cu^{I} and Cu^{II} binding sites but exhibiting no aggregation propensity (ref. 2). Hence for the aggregation study, the full-length Aβ40 peptide was used (See Supporting Information for details).

⊥ This is due to the fact that when the XANES data were recorded, we didn't pay attention of the time necessary for the Cu(II) reduction, which is quite long (see Supporting Info, Figure S1) and thus a higher PTA equivalents number accelerates the reduction reaction.

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