Interactions between copper (II) and β-amyloid peptide using capillary electrophoresis–ICP–MS: Kd measurements at the nanogram scale

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Interactions between copper (II) and β-amyloid peptide using capillary electrophoresis-ICP-MS: Kd measurements at the nanogram scale

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Abstract: Although the interaction between the β-amyloid peptide and copper (II) appears to play an important role in Alzheimer's disease, the affinity constant is still controversial and values are ranging from 10^7 M^{-1} to 10^{11} M^{-1}. With the aim of clarifying this point, a complementary method, based on the capillary electrophoresis-ICP-MS hyphenation, was developed and competitive binding experiments were conducted in the presence of nitrilotriacetic acid. The effect of the capillary surface (neutral or positively charged) and nature of the buffer (Tris or Hepes) have been studied. Tris buffer was found to be inappropriate for such determination as it enhances copper (II) complexes dissociation, already occurring in the presence of an electric field in capillary electrophoresis. Using Hepes, a value of 10^{10} M^{-1} was found for the affinity of the small β-amyloid peptide 1-16 for copper (II), which is in agreement with the values obtained for other proteins involved in neurodegenerative diseases. These constants were also determined in conditions closer to those of biological media (higher ionic strength, presence of carbonates).

Keyword: capillary electrophoresis / inductively coupled plasma mass spectrometry / binding constant / amyloid interaction / copper (II) complexes

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, mainly characterized by a progressive loss of memory and certain cognitive functions. Neuronal degeneration, initially located in the hippocampus, slowly spreads throughout the patient's brain. Although very widespread throughout the world, there is as yet no curative treatment for this disease. Thanks to numerous studies on the subject, the main markers of the disease are now well known.[1] Among them, the amyloid-β peptide (Aβ) is strongly involved in the development of AD and particularly $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments. $A\beta_{1-42}$, in its monomeric form is considered as nontoxic. However it is very prone to aggregation, forming oligomers, soluble protofibrils, and eventually fibrillar aggregates that deposit as amyloid plaques in the brain. Even if fibrillary aggregates have long been considered as the toxic species [2], evidences that soluble oligomeric species [3-6], including dimers and trimers [7], are key intermediates for toxicity, are now reported.

There is now a consensus about the neuronal toxicity of Aβ oligomers, and the important role of metal ions dyshomeostasis in AD, especially copper (Cu),[8, 9] and Aβ - Cu(II) complexes seem to play a crucial role in Aβ peptides' aggregation and toxicity.[10–13] In this context, intensive research effort have focused on the study of Aβ - Cu(II) complex, as reported by Faller *et al*.[14] Owing to the propensity of the peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ to self-aggregate, most studies have focused on the short peptide $A\beta_{1-16}$, which was proved to contain the high affinity site for copper complexation.[15] Even if the structure of the complex at pH 7.4 is well defined, there is much controversy regarding the affinity of Cu(II) for $A\beta_{1-16}$ and the reported constant values for the $A\beta_{1-16}$ - Cu(II) complex vary significantly.[16–19]

I. Zawisza *et al* [17] discussed the influence of the concentration ranges used with the classical biophysical techniques on the affinity constant $K_{A\beta1-16\ -\ Cu(II)}$. Values compiled by these authors were found to be around 10^7 M^{-1} using tyrosine fluorescence, 10^9 - 10^{11} M^{-1} depending on the competitor using isothermal titration calorimetry and 5 - $8x10^{10}$ using potentiometry. They proposed the formation of either dimeric complexes at high concentration or ternary complexes with competitors to explain the highest values. Alies *et al.* evoked

an underestimated contribution of the inner-filter effect using tyrosine fluorescence quenching and recalculated a Cu(II) affinity for $A\beta_{1-16}$ in the 10^{10} M^{-1} range.[19]
The situation becomes even more intricate considering the influence of buffers on the determination of the affinity constant. The nature and concentration of the buffer used seems to have an important impact on the determination of $K_{A\beta1-16 - Cu(II)}$. In fact, the competitive interactions, which may occur between the metal and some buffers, i.e. Tris and Hepes, seem to influence the value of the constant, determined with the conventional methods cited above. In order to take into account competitive interactions with the buffer, apparent constants are commonly converted into conditional constants using the affinity constants of Cu(II) - buffer complexes.[20]
Moreover, a Aβ - Cu(II) - Tris ternary complex was identified by TEM and ESI-MS. Therefore, the decrease of Cu(II) affinity for $A\beta_{1-16}$ and $A\beta_{1-42}$, observed by comparison with other buffers was not attributed to a competition with Tris but to the formation of these complexes.[21] The formation of ternary complexes involving Cu(II), Aβ and buffers/competing ligands was also highlighted by Rózga *et al.*[22].
Given these conflicting results regarding the affinity of Cu(II) for the $A\beta_{1-16}$ peptide, it seems important to provide additional experimental data in this area. The determination of the constant is of the utmost importance, not only to help deciphering the mechanisms related to AD that occur in the synapses, but also for the design of new copper (II) chelators for therapy. In this context, we propose to use a new analytical technique in this field, i.e. capillary electrophoresis hyphenated to ICP-MS, an elementary detection allowing to observe changes linked to the metal. This method will be applied to the determination of the affinity constant of $A\beta_{1-16}$ for Cu(II) in Tris and Hepes buffers, which represent the most studied buffers. Since these buffers are complexing ligands, competitive experiments will be performed using nitrilotriacetic acid (NTA) as competitor.

2. Materials and methods

2.1 Products and stock solutions

All product were used without further purification.
Tris (Trizma® base), Hepes, NaCl, $NaHCO_3$ and hydrochloric acid 37% from Sigma-Aldrich were used for buffer preparation. Four different buffers (10 mM Tris or Hepes containing 15 mM NaCl,, 10mM Hepes containing 100 mM NaCl in the presence or not of 30 mM sodium bicarbonate) were all adjusted at pH 7.4 by HCl addition.
NaOH 1M (Agilent), used for the capillary conditioning, and hydroxypropylcellulose (HPC) and hexadimethrine bromide (polybrene) used for capillary surface modification were also purchased from Sigma. Nitric acid 1% obtained by dilution in ultrapure water of HNO_3 65% (Merck, Suprapur® quality) was used for rinsing the capillary.
Acetone, 0.1% in water, and tributylphosphine (TBP), 0.03% in DMSO 2%, used as electroosmotic flow markers in CE_UV and CE-ICP-MS experiments respectively, and EDTA diluted in the sheath liquid buffer, were purchased from Sigma.
Lyophilized powder $A\beta_{1-16}$ (purity ≥ 95%) was purchased from GeneCust (Boynes, France).
Stock solutions of $A\beta_{1-16}$ *ca* 250 µM were prepared in the different buffers The exact concentration of $A\beta_{1-16}$ was determined by using UV measurements (Thermo Scientific™ NanoDrop™ UV-Vis Spectrophotometer) at 280 nm (ε = 1490 $M^{-1}cm^{-1}$, from UniProtKB/Swiss-Prot Database).
Stock solutions of $CuCl_2$ at 50 µM both buffers were obtained by dissolution of $CuCl_2.2H_2O$ purchased from Merck.
Stock solution of nitrilotriacetic acid (NTA) at 2.5 mM in both buffers obtained by dissolution of NTA purchased from Merck.

Samples were freshly prepared just before use from copper (II), NTA and $A\beta_{1-16}$ stock solutions diluted in the appropriate buffer in order to obtain final mixtures with the desired concentrations ([$A\beta_{1-16}$] = 100 µM, [Cu(II)] = 10 µM and 5 µM < [NTA] < 1500 µM).

2.2 CE-ICP-MS conditions

A Beckman P/ACE MDQ instrument was used for CE separations using 32 Karat Software for both control and UV data acquisition. The background electrolyte was either 10 mM Tris, 15 mM NaCl or in 10 mM Hepes, 15 mM NaCl, both at pH 7.4.
For the separation, fused silica capillaries (64 cm x 75 µm i.d., 375 µm o.d., Polymicro Technologies) have been pre-treated either with HPC to obtain a neutral surface or with polybrene to obtain a positively charged surface.
In the case of HPC, a thermal coating protocol, already described elsewhere [23], was applied using a HPC solution, 0.05 g/ mL in water. HPC capillaries were then rinsed twice with HNO_3 1% at 10 psi during 5 min and then conditioned with background electrolyte at 10 psi during 5 min before each separation. Separations were performed at 25°C at -7 kV assisted by a pressure of 0.3 psi.
Dynamic coating was performed by flushing the fused silica capillary with a polybrene solution at 0.1% m/m in the desired buffer for 30 min at 5 psi before the first separation and for 10 min at 5 psi between runs.
Separations were performed at 25°C at -15 kV. Before each separation, the capillary was conditioned with background electrolyte at 5 psi during 2 min.
In both cases, samples were introduced into the capillary by hydrodynamic injection under 0.5 psi during 5 sec (around 25 nL) and all experiments were performed in triplicate. At the end of the separation, a pressure rinse (5 psi) with HNO_3 1% was performed for 5 min to release the Cu(II) sorbed onto the capillary walls.
An Agilent 7700 ICP-MS equipped with a MicroMist nebulizer was used as detector and controlled by the MassHunter software. Copper was detected at m/z = 65 at the end of the capillary (64 cm). Operating conditions were: plasma gas flow rate: 15 $L.min^{-1}$, carrier gas flow rate: 1 $L.min^{-1}$, auxiliary gas flow rate: 0.9 $L.min^{-1}$, makeup gas flow rate: 0.1 $L.min^{-1}$, radiofrequency power 1550 W for the plasma. Other parameters were tuned in order to maximize the signal.
The CE-ICP-MS hyphenation was achieved via a home-made sheath-flow interface described elsewhere.[24] Electrical connection was achieved thanks to a platinum ground electrode positioned in one of its inlets. The sheath liquid was a 5 mM Tris solution adjusted at pH 7.4 and containing 10 µM EDTA, and was introduced by self-aspiration to another inlet of the interface.

2.3 Determination of the binding constants

Samples were freshly prepared from copper (II), NTA and $A\beta_{1-16}$ stock solutions diluted in the appropriate buffer in order to obtain final mixtures with the desired concentrations ([$A\beta_{1-16}$] = 100 µM, [Cu(II)] = 10 µM and 5 µM < [NTA] < 1500 µM). After incubation at room temperature for 2h, samples were analyzed in triplicate by CE-ICP-MS and copper percentage was calculated for each species. Since an experiment lasts around 30 min in HPC capillaries and 45 min in polybrene capillaries, the whole set of experiments, necessary to determine the complexation constant, requires at least 9 levels of NTA concentrations and was then realized over 2-3 days. In addition, two control experiments, using 100 µM and 250 µM NTA as competitor, were performed each day.
Complexation constants were then determined by fitting the experimental data with the theoretical curves by unweighted nonlinear least squares using the Origin 8.5 software, assuming the absence of free copper in the solution.

3. Results and discussion

3.1 Selection of a suitable copper (II) competitor

The determination of the affinity constant of $A\beta_{1-16}$ for Cu(II) was performed by competition assays. The selection of NTA as suitable competitive chelator was based on the following conditions (i) affinity constants of the two ligands must be of the same order of magnitude, (ii) the electrophoretic mobilities (μ_{ep}) of the copper (II) complexes must be different to achieve a good separation between the two complexes in CE-ICP-MS.

log $K_{NTA\text{-}Cu(II)}$ (pH 7.4) was calculated from the log $\beta_{NTA\text{-}Cu(II)}$ value of 12.94 recommended by the IUPAC and recalculated as an apparent value at pH 7.4 thanks to the pKa value of the tertiary amine of NTA equal to 9.66.[25] On the basis of the apparent affinity constants reported in literature at pH 7.4 for the $A\beta_{1-16}$ - Cu(II) complex ($7 < \log K_{A\beta 1\text{-}16\text{-}Cu(II)}$ (pH 7.4) < 12), log $K_{NTA\text{-}Cu(II)}$ (pH 7.4) = 10.68 appears to be in the appropriate range of affinity.

Mobilities of both complexes were determined thanks to affinity capillary electrophoresis (ACE) experiments, using acetone as electroosmotic flow marker. For that purpose, experiments were carried out by injecting $A\beta_{1-16}$ and NTA individually and separating them in Tris 10 mM electrolytes containing increasing amounts of copper (II). Mobilities of free ligands were measured in the absence of copper and mobilities of the copper (II) complexes were measured in the presence of a high excess of copper (II), so that the ligands were saturated (see SI). Table 1 listed the obtained electrophoretic mobilities of both ligands either free or complexed and showed that the mobilities of the two complexes were quite different.

Table 1 *Electrophoretic mobilities of free and complexed ligands determined by affinity capillary electrophoresis*

Compounds	$A\beta_{1-16}$	$A\beta_{1-16}$ - Cu(II)	NTA	NTA - Cu(II)
μ_{ep} ($m^2.V^{-1}.s^{-1}$)	$-8x10^{-9}$	$-1x10^{-8}$	$-5x10^{-8}$	$-2.5x10^{-8}$

3.2 Determination of suitable conditions to limit dissociation

Complexes dissociation due to disequilibrium effects is one of the major issue when using capillary electrophoresis and it might affect the determination of affinity constants. Indeed, as separation occurs, ligands and metals are no longer in equilibrium and complexes may start to dissociate. Consequently, maintaining the integrity of metal-ligands complexes during the separation is an important factor.

If metal and ligand dissociated from the metallic complex when separation occurs and may rebind only as long as they are not removed from the interaction zone. The deleterious effect is not only related to the difference in mobilities but also to the electric field strength and this should be kept as low as possible. Therefore, CE separation conditions were investigated to avoid any modification of the equilibrium by the application of the voltage.

Dissociation of the complexes was investigated by comparing the separations performed for different conditions. 100 µM $A\beta_{1-16}$ and 100 µM NTA with 10 µM Cu(II) solutions were injected and separated in either Tris or Hepes electrolyte using different applied voltages (-7 to -20 kV). Additionally, capillary wall effects were examined by using either HPC or polybrene capillary.

Copper(II) areas of the remaining complexes were measured using ICP-MS. In order to overcome the small variations due to the use of solutions of copper (II) complexes from different batches, values were expressed as ^{65}Cu area/reference area, where ^{65}Cu area represents the area measured for a given voltage and the reference area, the mean area measured for -7 kV. The values obtained are presented in figure 1.

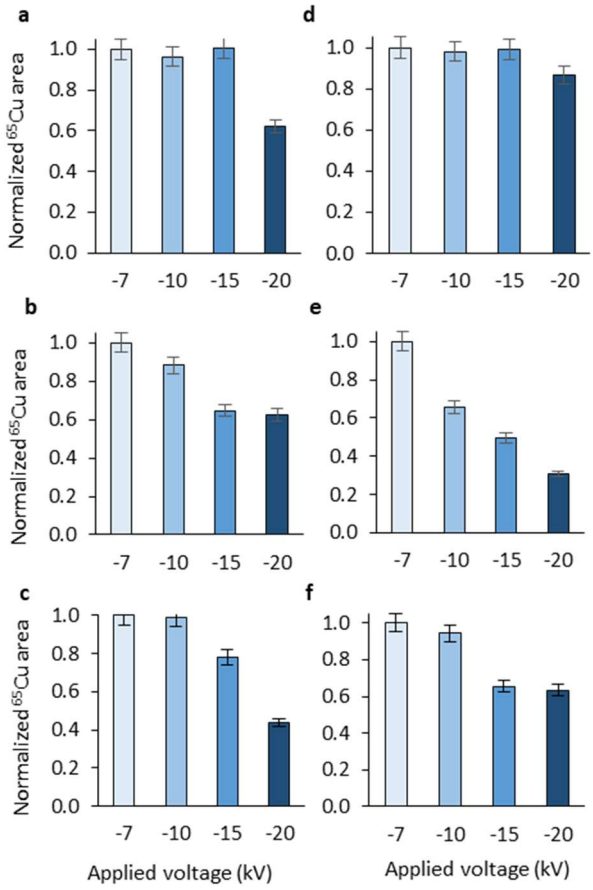

Fig. 1 *Voltage effect on Aβ - Cu(II) (a,b,c) and on NTA - Cu(II) (d,e,f) recoveries with Tris buffer and polybrene capillary (a,d) or Tris buffer and HPC capillary (b,e) or Hepes buffer and HPC capillary (c,f)*
Experimental conditions: HPC or polybrene coated - capillary (75 µm i.d. x 64 cm), 10 mM Tris or 10 mM Hepes buffer, 15 mM NaCl at pH 7.4, 25°C. Detection: ICP-MS at m/z = 65 at the end of the capillary
Separations in HPC capillary were carried out with an assisted pressure of 0.3 psi

In the case of polybrene-coated capillary (fig. 1a and d), data showed a non significant effect of the voltage applied on the copper peak area for both complexes, except at -20 kV.
On HPC capillary (fig. 1b, c, e, and f), a clear decrease of copper area is observed as a function of the applied voltage, indicating an influence of the capillary inner surface. The occurrence of an adsorption of copper (II) onto HPC is then assumed, such a polysaccharide having already been reported in the literature as an efficient material for removing metals from aqueous solutions.[26]
Moreover, this effect seems to be more pronounced for NTA - Cu(II) than for $A\beta_{1-16}$ - Cu(II) complex, suggesting that NTA - Cu(II) is more prone to dissociation. This could be attributed to the significant difference in mobilities observed between free and complexed NTA (table 1), leading to a faster escape of this ligand from the complex zone hence decreasing the rebinding probability.
Finally, the comparison of the results obtained with HPC using Tris and Hepes electrolytes clearly indicates that the nature of the electrolyte influences the dissociation. Results clearly show that the presence of Tris seems to promote the dissociation of the NTA - Cu(II) complex in HPC capillary.
The difference observed between Tris and Hepes buffers could be explained by the presence of competitive interactions, greater with Tris than with Hepes. Tris is indeed known to interact with Cu(II), forming 1:1 and 2:1 complexes with affinity constants of 5.78×10^3 M^{-1} and 4.46×10^6 M^{-2} respectively.[27] Hepes buffer is a weaker competitor but also associates with Cu(II) forming a 1:1 complex with an affinity constant of the order of 10^3 M^{-1}.[28] These interactions may contribute to the NTA - Cu(II) dissociation provoked by field effect. However, the formation of a more stable ternary complex NTA - Cu(II) - Hepes cannot be excluded as reported

by Haas *et al.*[29]. The formation of such a complex could lead to the opposite effect, i.e. decreasing the dissociation of the NTA - Cu(II) in Hepes. However, a less pronounced but similar trend was observed for the $A\beta_{1\text{-}16}$ - Cu(II) complex, for which it was reported that Hepes is not involved in a ternary complex.[20]

Consequently, the voltages applied during the competition separations described hereafter will be -7kV with assisted pressure of 0.3 psi to counterbalance the absence of electroosmotic mobility (μ_{eo}) in the HPC capillary and -15kV in the polybrene capillary.

3.3 Determination of $A\beta_{1\text{-}16}$ - Cu(II) affinity constant

A competitive study was then conducted in order to compare the affinity of $A\beta_{1\text{-}16}$ and NTA for Cu(II).
For this purpose, various solutions containing 10 µM Cu(II), 100 µM $A\beta_{1\text{-}16}$ and increasing concentrations of NTA were analyzed using CE-ICP-MS after 2h of incubation at room temperature. Experiments were performed in both buffers (either Tris 10mM or Hepes 10 mM, NaCl 15 mM at pH 7.4) in different capillaries to investigate the influence of the buffer on the constant determination.
For each series of experimental conditions, the distribution of Cu(II) between the two species was calculated. The high excess of ligands over copper (II) ensures the existence of the sole 1:1 complexes.
Assuming a 1:1 stoichiometry for both copper (II) complexes, the competition can be described by the following simplified equilibrium:

$$A\beta_{1\text{-}16} \text{ - Cu(II)} + \text{NTA} \leftrightarrows \text{NTA - Cu(II)} + A\beta_{1\text{-}16}$$

where charges are voluntarily omitted.
Equation 1 defined the equilibrium constant K:

$$K = \frac{[\text{NTA - Cu(II)}][A\beta_{1\text{-}16}]}{[A\beta_{1\text{-}16}\text{ - Cu(II)}][\text{NTA}]} = \frac{K_{\text{NTA - Cu(II)}}}{K_{A\beta 1\text{-}16\text{ - Cu(II)}}} \qquad \text{(equation 1)}$$

$K_{\text{NTA - Cu(II)}}$ being the affinity constant of NTA for Cu(II) and $K_{A\beta 1\text{-}16\text{ - Cu(II)}}$, the affinity constant of $A\beta_{1\text{-}16}$ for Cu(II).
From equation 1 and the different mass balance equations, it can be shown that the percentages of the two species can be related to the NTA concentration by a quadratic polynomial law.
Therefore, the affinity constant $K_{A\beta 1\text{-}16\text{ - Cu(II)}}$ was obtained by adjustment of the curves by nonlinear least-squares analysis assuming the sole formation a 1:1 complex.

3.3.1 $A\beta_{1\text{-}16}$ - Cu(II) affinity constant in Tris

Experiments were first conducted in Tris buffer. The distribution of Cu(II) between the different species as a function of NTA concentration as well as a few representative mobilograms are shown in figure 2, for both capillaries. The electropherograms were converted into mobilograms by plotting the signal as a function of the electrophoretic mobility, instead of the recorded time. For that purpose, the electrophoretic mobilities were calculated by subtracting the migration contribution associated to neutral species to the apparent migration of the solutes. The μ_{ep} were determined using TBP as a neutral marker, thanks to equation 2**:**

$$\mu_{ep} = \frac{L_d L_t}{E} * \left(\frac{1}{t_m} - \frac{1}{t_{NM}}\right) \qquad \text{(equation 2)}$$

where L_d is the length of detection, L_t is the total length of the capillary, V is the applied voltage, t_m is the migration time of the analyte peak and t_{NM} is the migration time of the neutral marker peak.

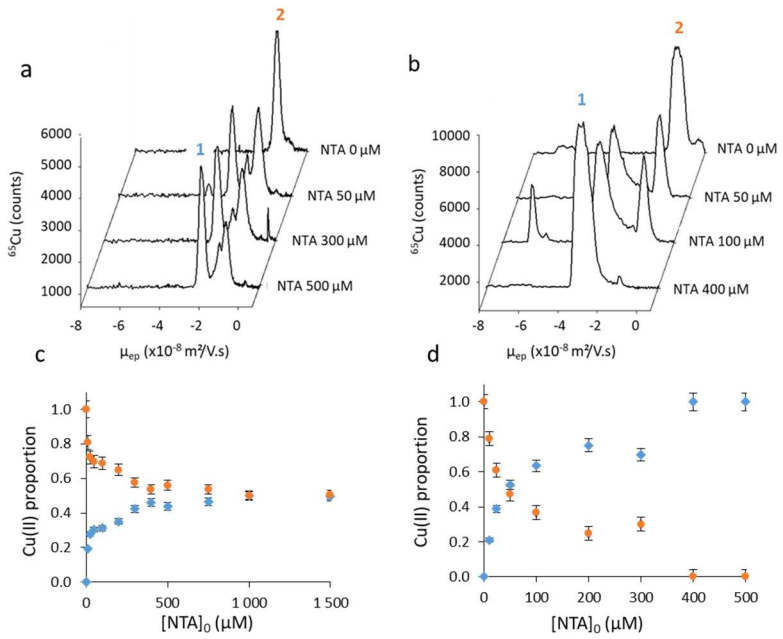

Fig. 2 *Competition between Aβ$_{1-16}$ and NTA for Cu(II) binding in Tris buffer*
Peaks 1 and 2 represent NTA - Cu(II) and Aβ$_{1-16}$ - Cu(II) complexes respectively
(a,b): Mobilograms obtained from CE-ICP-MS separations of 88 µM Aβ$_{1-16}$ and 10 µM Cu(II) with various concentrations of NTA in HPC or polybrene capillary respectively
(c,d): Cu(II) proportion in Aβ$_{1-16}$ - Cu(II) (●) or NTA - Cu(II) (◆) complexes in HPC or polybrene capillary respectively as a function of NTA concentration
Experimental conditions: HPC or polybrene coated - capillary (75 µm i.d. x 64 cm), 10 mM Tris buffer, 15 mM NaCl at pH 7.4, 25°C. Detection: ICP-MS at m/z = 65

Since the bound Cu(II) does not alter the migration order as shown by affinity capillary electrophoresis experiments, the first main copper (II) complex peak was assigned to NTA - Cu(II) and the second one to Aβ$_{1-16}$ - Cu(II). Cu(II) is mainly distributed between Aβ$_{1-16}$ and NTA, only a negligible fraction being eluted during the nitric acid washing step (data not shown). However, the presence of a tailing NTA - Cu(II) peak in the polybrene capillary (fig. 2b) or the appearance of a third peak in the HPC capillary (fig. 2a) can be observed. These observations may be explained by the dissociation of copper (II) from the NTA - Cu(II) complex, as already discussed in paragraph 3.2.
In the case of HPC, the dissociation is likely to be accompanied by an adsorption of copper (II) onto the HPC capillary. However, examination of the total copper areas, whatever the NTA concentration, led to the conclusion that this adsorption was reversible, if any, and that the free Aβ$_{1-16}$ was able to rebind this copper (II) released by NTA (fig. 2a and c). Such an assumption precludes the calculation of $K_{Aβ1-16 - Cu(II)}$ in such conditions. As can be seen in fig. 3, attempts to fit experimental points with a quadratic model of competitive equilibria were unsuccessful.
In the polybrene capillary, the free copper (II) removed from the complex zone starts its migration towards the cathode. Taking this dissociation during electromigration into account, log $K_{Aβ1-16 - Cu(II)}$ was assumed to be estimated but will suffer from strong uncertainty. Using a quadratic model (fig. 3), a bad correlation between experimental values and fitted values was obtained. However, log $K_{Aβ1-16 - Cu(II)}$ was estimated to about 10.

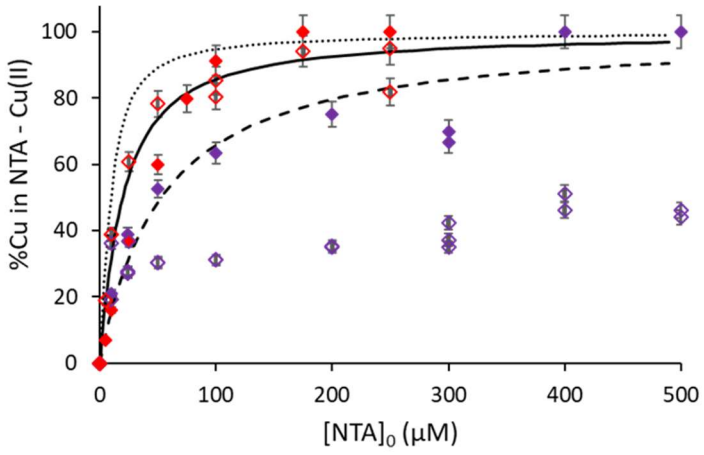

Fig.3 *Experimental data for competition effect in HPC with Tris (◇), in HPC with Hepes (◇), in polybrene with Tris (◆), or in polybrene with Hepes (◆) and theoretical data for log $K_{Aβ1-16 - Cu(II)}$ = 9.4 (⋯⋯), log $K_{Aβ1-16 - Cu(II)}$ = 9.9 (—), and log $K_{Aβ1-16 - Cu(II)}$ = 10.4 (- - -)*

3.3.2 $Aβ_{1-16}$ - Cu(II) affinity constant in Hepes

Experiments were then conducted similarly in Hepes and led to the separation of two well-resolved peaks, whatever the NTA concentration. Representative electropherograms in Hepes, treated using equation 2, and percentages of the copper (II) complexes as a function of NTA concentration are presented in figure 4.

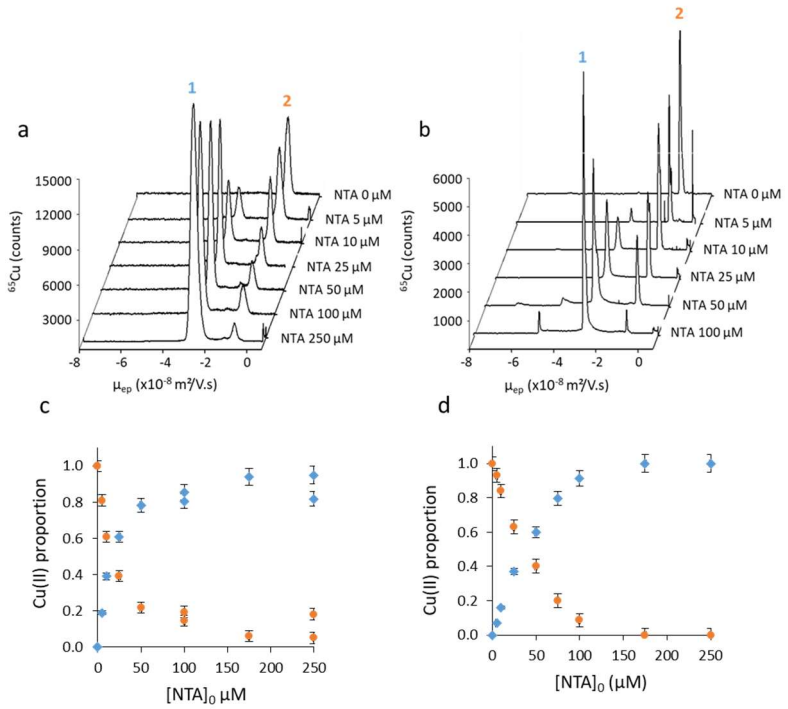

Fig.4 *Competition between $Aβ_{1-16}$ and NTA for Cu(II) binding in Hepes buffer*
Peaks 1 and 2 represent NTA - Cu(II) and $Aβ_{1-16}$ - Cu(II) complexes respectively
(a,b): Mobilograms obtained from CE-ICP-MS separations of 100 µM $Aβ_{1-16}$ and 10 µM Cu(II) with various concentrations of NTA in HPC or polybrene capillary respectively
(c,d): Cu(II) proportion in $Aβ_{1-16}$ - Cu(II) (●) or NTA - Cu(II) (◆) complexes in HPC or polybrene capillary respectively as a function of NTA concentration
Experimental conditions: HPC or polybrene coated - capillary (75 µm i.d. x 64 cm), 10 mM Hepes buffer, 15 mM NaCl at pH 7.4, 25°C. Detection: ICP-MS at m/z = 65

Using both capillaries, experimental data (fig. 4c and d) were successfully fitted with a quadratic model (fig. 3) and log $K_{A\beta 1\text{-}16\text{ - }Cu(II)}$ was found to be (9.9 ± 0.2) and (9.85 ± 0.06) in polybrene and HPC capillary respectively. However, using NTA as a competitive chelator in Hepes buffer, the formation of a ternary complex with NTA - Cu(II), i.e. NTA - Cu(II) - Hepes, cannot be excluded. These interactions should result in an apparent $K_{A\beta 1\text{-}16\text{ - }Cu(II)}$ affinity constant value that would appear weaker than the actual conditional constant value at pH 7.4. However, this ternary complex may not decrease the affinity constant by several orders of magnitudes. For instance, Rózga *et al.* reported an apparent constant lowered by a factor of 4 (log K = 0.6), as a result of the competition from the ternary NTA - Cu(II) - Hepes complex.[25]
Table 2 recapitulates the affinity constants obtained when fit was possible.

Table 2: Affinity constants of $A\beta_{1\text{-}16}$ - Cu(II) complexes using various buffers and capillary surfaces

Capillary surface	Buffer	$[A\beta_{1\text{-}16}]_0$ (µM)	$[NTA]_{50}$ (µM)	log K
Polybrene	10 mM Tris	88	35	*ca* 10
Polybrene	10 mM Hepes	100	16	9.9 ± 0.2
HPC	10 mM Hepes	100	15	9.85 ± 0.06

Under the three conditions (Polybrene / Tris, Polybrene / Hepes, and HPC / Hepes), log $K_{A\beta 1\text{-}16\text{ - }Cu(II)}$ was found to be around 10, similar to that of synuclein, involved in Parkinson disease, but lower than that of albumin by 2 orders of magnitude.

3.4 Influence of the incubation medium on $A\beta_{1\text{-}16}$ - Cu(II) affinity constant

Since biological media often contain high concentrations of salts, experiments were then performed on samples containing either 10 mM Hepes, 100 mM NaCl or 10 mM Hepes, 100 mM NaCl and 30 mM sodium bicarbonate. However, these high concentrations of salts are not adapted to capillary electrophoresis analyses, as they generate high currents, which induce an increase of temperature in the capillary due to Joule effect. Consequently, the separations were performed in HPC capillaries using 10mM Hepes, 15 mM NaCl as electrolyte. These conditions were previously found to allow the determination of complexation constants in a faster way than using polybrene capillaries.
A comparison of the distribution of copper (II) between the two species ($A\beta_{1\text{-}16}$ and NTA) in the different sample matrices is given in Figure 5.

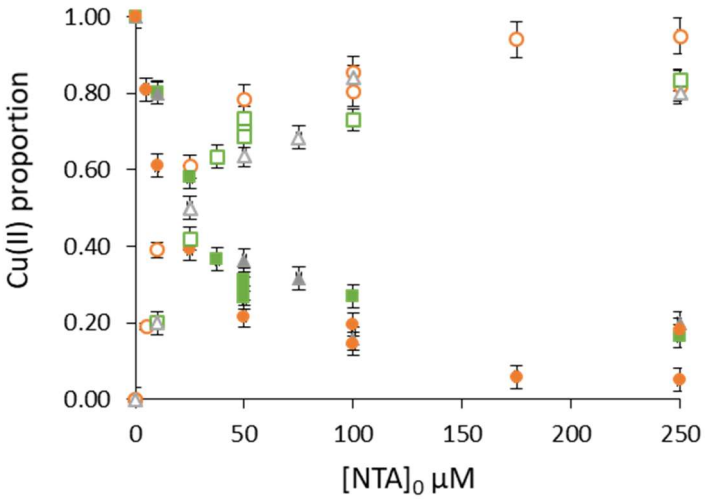

Fig.5 *Cu(II) proportion in $A\beta_{1\text{-}16}$ - Cu(II) (plain symbols) or NTA - Cu(II) complexes (empty symbols) in 10mM Hepes, 15 mM NaCl, (●), 10 mM Hepes, 100 mM NaCl (■) 10 mM Hepes, 100 mM NaCl, 30 mM $NaHCO_3$ (▲) as a function of NTA concentration*

Experimental conditions: HPC coated - capillary (75 µm i.d. x 64 cm), 10 mM Hepes buffer, 15 mM NaCl at pH 7.4, 25°C, separation at -7kV + 0.3 psi. Detection: ICP-MS at m/z = 65

Figure 5 does not exhibit any significant differences between the three samples. The calculation of log $K_{Aβ1-16\text{-}Cu(II)}$ gives values of (10.07± 0.03) and (10.13± 0.05) in 100 mM NaCl and 100 mM NaCl, 30 mM $NaHCO_3$ media, respectively.

This very slight increase, compared to the value obtained in a 10 mM NaCl medium, could be due to the neglected ion-pairing capacity of NTA with Na^+ in the calculation. Moreover, the similarity with the value obtained in the carbonated medium seems to indicate that none of the two complexes formed ternary complexes involving carbonates. Most important is the absence of formation of carbonato complexes at pH 7.4, as ascertained by the nearly perfect superposition of the electrophegrams obtained in both media (see SI). This suggests that copper (II) is totally recovered in both NTA and $Aβ_{1-16}$ complexes and that the formation of very labile and dissociated Cu(II) complexes might be exclude. This was indeed expected, taking into account the reported complexation constants for these species: log K_{CuHCO3} = 1.82, log K_{CuCO3} = 6.73, and log $K_{Cu(CO3)2}$ = 10.41. [30] All together, the results seem to indicate that the formation of Aβ - Cu(II) at high concentrations of carbonate is relevant and has to considered in biological media.

4. Conclusion

As a conclusion, the affinity constant between copper and $Aβ_{1-16}$ was determined by competition experiments, and can be extended to other small ligands exhibiting very different electrophoretic mobilities from their complexes. Nevertheless, since the described method is a fit-to-purpose method, the conditions will have to be adapted as a function of the lability of the complexes.Owing to the small volumes required by CE-ICP-MS (around 25 nL per injection in this study), it offers the possibility to monitor copper distribution using less than 50 pmol of $Aβ_{1-16}$. Moreover, the concentrations in the 100 µM range allow a direct comparison with fluorescence, regarding to this parameter. The value determined for the constant of $Aβ_{1-16}$ - Cu(II) at pH 7.4, in the 10^{-10} M range, is in agreement with previous determinations performed using ITC and potentiometry and validate a possible bias observed in fluorescence, as described by Allies *et al.*[19]. However, experimental conditions should be carefully selected to avoid misinterpretation of the data. Although Tris was often recommended for classical biophysical studies at equilibrium, this buffer was found to enhance dissociation in capillary electrophoresis, presumably because of its higher competing power. As a consequence, affinity constants cannot be determined accurately in this buffer. Hepes was found to be more adequate and log $K_{Aβ1-16\text{ - }Cu(II),}$ determined using both HPC and polybrene capillaries, were identical.

Moreover, in that range of concentrations, Aβ - Cu(II) complex still exists at high concentrations of carbonates, close to those encountered in biological media and no formation of carbonato complexes was noticed at pH 7.4.

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