How very small changes in the peptide sequence of β -amyloids influence their redox properties. Electrochemical studies of $A\beta(11-16)$ and $pA\beta(11-16)$ in two different electrolytes.

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Abstract:

HERE WE DEMONSTRATE A SIGNIFICANT DIFFERENCE IN REDOX BEHAVIOUR OF COPPER COMPLEXES WITH β -AMYLOIDS $AB(11-x)$ AND $PAB(11-x)$ WHICH ARE MODELS FOR IMPORTANT COMPONENTS OF SENILE PLAQUES. A SMALL CHANGE IN THE PEPTIDE CHAIN MAY ENHANCE REACTIVE OXYGEN SPECIES (ROS) FORMATION WHICH CAN SEVERELY DAMAGE NERVE CELLS.

Introduction

Alzheimer's disease (AD) is currently the most common neurodegenerative disease, affecting 10% of people over the age of 65 and more than 30% over the age of 85. However, an ageing society will lead to a dramatic increase in the number of AD cases in the future. According to current estimates, the number of older people with AD will have tripled by 2050 and reach 131.5 million worldwide. Although AD was first described more than 100 years ago, it remains incurable. According to one of the leading hypotheses, overproduction or excessive aggregation of peptides from the β -amyloid ($A\beta$) group is responsible for the development of AD. Additionally, research over the last twenty years has provided strong evidence for the importance of oxidative stress in the pathogenesis of Alzheimer disease. [1] The initial source of the reactive oxygen species (ROS) production is highly dependent on redox-active transition metals, mainly copper(II) ions, which create complexes with $A\beta$. [2] Until recently, the majority of chemical studies on the formation and properties of complexes concerned mainly the "canonical" $A\beta(1-x)$ (where $x = 16, 28, 40$ and 42) peptides. The use of spectroscopic and electrochemical techniques [3] allows not only to describe the structure of the $Cu(II)A\beta(1-x)$ complexes but also to determine the binding constant for $Cu(II)$ ions, which at physiological pH (7.4) is $10^{10} M^{-1}$. [4] It was discovered that $Cu(II)$ ions in $Cu(II)A\beta(1x)$ facilitate the

production of ROS. The pathway of the Cu(II)-A β /Cu(I)-A β process was investigated directly using voltammetric techniques [5–8]

It is worth noting that a several studies show that A β (1-x) is not the only isoform of A β present in the brains of AD patients. Many proteases and peptidases like neprilysin (NEP) insulin-degrading enzyme (IDE), endothelin-converting enzyme or angiotensin-converting enzyme to cleave A β (1-x) to N-terminally altered isoforms.[9,10] N-truncated A β s exhibit very different properties of binding Cu(II) ions compared with A β (1-x), which is strongly connected to their amyloid sequence. The A β (5–16) peptides possess a His-2 motif in which Cu(II) ions bind to XH by three nitrogen atoms (3N) from the terminal amine, the first amide group and the imidazole ring. The emergence of the 3N binding mode results in higher stability of the complex for Cu(II)-A β (5–16) ($K=9.55 \times 10^{12} \text{ M}^{-1}$ at pH 7.4) compared to Cu(II)-A β (1-16) complexes.[11] Cu(II) ions binding to A β (5–16) undergo both Cu(II)/Cu(III) oxidation and Cu(II)/Cu(I) reduction processes. However, irreversible oxidation to Cu(III) could be registered only at potentials around 1 V and higher (all potentials referred to *vs* Ag/AgCl), and therefore this process is biologically irrelevant. Nevertheless, for Cu(II)/Cu(I), a pair of peaks appears at -0.680 V (cathodic process) and 0.200 V (anodic process), respectively, which may enhance the oxidative stress and ROS generation. [11,12]

The process of enzymatic degradation of A β (1-x) at positions 4, 11 and 12 also leads to the production of a peptide family with very high affinities for Cu(II) ions (stability constant $\sim 10^{14} \text{ M}^{-1}$). [13–16] Due to the presence of an XYH sequence at their N-terminus, called an amino-terminal copper and nickel motif (in short ATCUN), Cu(II) ions are coordinated in a square-planar geometry, which stabilises the Cu(II) and the Cu(II)/Cu(III) redox process is seen at a potential of $\sim 0.8 \text{ V}$. [8,13] According to a recent report, Cu(II) bonded by A β (4-16) and A β (11-16) can undergo reduction to Cu(I) as well, however, at very low potentials $< -1 \text{ V}$, which makes these complexes redox silent.[4]

Among ATCUN-type beta amyloids, the ones whose amino acid sequence begins with glutamic acid (1-letter abbreviation: E), like A β (11-42), are particularly interesting. The level of A β (11-42) in AD brain plaques is comparable to A β (1-42) and can be as much as 18,6%.[17] Glutamic acid in A β (11-42) undergoes dehydration catalysed by glutaminy cyclase to form cyclic pyroglutamate, resulting in a new derivative pA β (11-42).[18] The condensation of E amino acid to pE removes the ATCUN type binding properties resulting in a dramatically reduced stability constant ($K=5,8 \times 10^9 \text{ M}^{-1}$).[16] Moreover, this pA β (11-42) and a similar

pyroglutamate amyloid, pA β (3-42), play crucial roles in AD development due to a combination of several factors, including that the pE residue stabilises the peptides against further degradation by enzymes and a tendency to fast formation of toxic oligomers. [19,20] It is also important that pE-peptides appear very early in the disease process, and according to data are the major component of senile plaques (contribution up 50%)[21]. Binding properties and Cu(II) complexes of A β (11-16) as well as of pA β (11-16) were characterised by many techniques. [4,14,16,22,23] However, no studies have been performed on the redox activity of Cu(II) ions bounded by pA β (11-x), even though pyroglutamate-modified A β s are among the most important components of all N-terminal truncated species. [17,24] To provide a more comprehensive understanding of AD pathophysiology, this knowledge gap should be filled. Therefore, we aim to characterise the electrochemical properties of complexes of Cu(II) ions with A β (11-x) and pA β (11-x) peptides by using A β (11-16) and pA β (11-16) as a suitable, well-soluble model. The Cu(II)-A β complexes were studied not only in solutions of physiological pH (7.4) but also in lower and higher pH, which allowed to better characterise the influence of coordination forms on the redox processes. The studies were performed in two different solutions, 4 mM HNO₃/96 mM KNO₃, commonly used in potentiometric experiments with peptides, and 0.1 M phosphate buffer. Because phosphate anions may influence the Cu(II)-peptide species, the latter buffer better reflects the conditions in the human body. [12,25]

Here, the redox behaviour of Cu(II) complexes with A β (11-16) (peptide sequence: EVHHQK-am) and its pyroglutamate counterpart pA β (11-16) (sequence pEVHHQK-am) was investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) in two kinds of solutions: 4 mM HNO₃/96 mM KNO₃ and 0.1 M phosphate buffer. A β (11-16) belongs to the ATCUN peptide family, and therefore, the Cu(II) ion is bonded via four nitrogens by the EVH sequence (creating 4N complexes) in a square-planar geometry.[4] According to Bossak-Ahmad et al.[16] in the pH range from ~4 to 11, four stoichiometric forms, differing in the protonation of the side chain, could be detected for the single 4N binding mode using UV-Vis, CD spectroscopy and potentiometry. In contrast, the pyroglutamyl modification in pA β (11-16) removes the Cu(II)-anchoring amine function leading to creation the less stable complexes. Therefore, pEVHHQK-am demonstrated the presence of several pH dependent Cu(II) complexes. In this work, we have performed experiments in solutions with physiological pH 7.4 but also at pH 6.2 in order to investigate the electrochemical response for 2N binding mode of pA β (11-16)-Cu(II) complexes, and pH = 9.0 where only the 4N structure is formed for this amyloid. The above values have been selected based on the species distribution diagrams[16]

(SI, Table 3) The most stable 4N coordination modes for both peptides are shown in Scheme 1.

Scheme 1. (A) 4N coordination mode of Aβ(11-16)-Cu(II), and (B) 4N coordination mode of pAβ(11-16)-Cu(II)

Comparison of redox properties of Cu(II) coordinated by Aβ(11-16) and pAβ(11-16) in potassium nitrate.

We used cyclic voltammetry to investigate the anodic as well as the cathodic electrochemical behaviour of Aβ(11-16) and pAβ(11-16) coordinated with 0.9 equiv Cu(II). (A small Cu(II) deficiency helps to avoid the interference from uncomplexed Cu(II) cations) (Fig. 1). On the first scan for Aβ(11-16)-Cu(II) complexes, no oxidation signal was detected in the range from -0.5 V to 0.6 V corresponding to the expected position of the Cu(I)/(II) process, while in the higher potential range (0.6 V to 0.9 V), the oxidation and reduction peaks related to the Cu(II)/(III) process were recorded. According to the literature data, the formal potential (E_f) for ATCUN type Cu(II) complexes {NH₂, 2×N⁻, Im(His)} should be close to 0.77 V vs. Ag/AgCl.[26] However, recent studies show that E_f depends on the peptide sequence and could be shifted to either more positive or negative potentials. [27–29] In this study, E_f and peak separation (ΔE) was determined as 0.730 V and 0.071V, respectively. Similarly, DP voltammograms (SI 1.1) revealed a one-electron oxidation step ($b_{1/2} = 0.098$ V) Cu(II)/Cu(III) at $E = 0.712$ V. Electrochemical parameters for Aβ(11-16)Cu-(II) in all studied pH are collected in Table 1 (SI). The obtained results are in good agreement with available literature Esmieu et al.[4] reported a similar quasi-reversible oxidation process of Aβ(11-16)Cu-(II) dissolved in 0.05 M phosphate buffer at pH 7.4. The formal potential in that case was shifted to more positive values. In our previous studies performed for Cu(II) complexes with Aβ(4-16) and its analogues no Cu(II)/Cu(I) reduction was observed down to the potential -0.8 V, which indicated redox silence of these Aβs. [8,13] However, Cu(II) bonded to Aβ(4-16) or Aβ(11-16) can be reduced to Cu(I) but at a much lower potential value, which is not relevant for condition in the human body. The reorganisation from 4N square planar coordination of the ATCUN motif to the linear coordination requires a large amount of energy. Therefore, we have observed the irreversible and broad reduction peak at very low potential ~ - 1.14 V vs. Ag/AgCl

(Fig. 1, A and B). Interestingly, the new, reduced $A\beta(11-16)$ -Cu(I) species can be electrochemically re-oxidised to $A\beta(11-16)$ -Cu(II) at the potential ~ 0.2 V (second scan, Fig. 1 B), and this process is pH dependent. At higher pH (9.0) oxidation occurs more easily (at lower potential values) (SI, Table 1) than in lower pH (6.2). Comparison of scan 1 with scan 2 shows that the oxidation of Cu(I)/Cu(II) occurs only if the metallic center is reduced at potentials lower than -1 V.

To compare the electrochemical behavior of both peptide Cu(II) complexes, we performed experiments for $pA\beta(11-16)$ -Cu(II) in the same potentials range as for $A\beta(11-16)$ -Cu(II) (Fig. 1C, D). The shape of the curves has changed significantly. The electrochemical behavior of Cu(II) bound to $pA\beta(11-16)$ -peptide is mainly influenced by the geometrical structures of the formed complexes. For $pA\beta(11-16)$ -Cu(II), at pH 6.2 two different mode exist: 2N and 4N, however the 2N mode dominates (see SI Table 3). On the basis on our [30] and literature data [31] two processes were predicted: Cu(I)/Cu(II) (for 2N structure) and Cu(II)/Cu(III) (for 4N structure). It is worth noting that the shapes of the recorded curves are similar both on the 1 and 2nd scan, scan, unlike for the $A\beta(11-16)$ -Cu(II). The first process, corresponding to the single electron Cu(I)/Cu(II) reaction, was more pronounced than the second anodic step (Cu(II)/Cu(III)). At pH 6.2 it was observed at $\approx +0.333$ V and a cathodic peak was observed at a potential of approximately +0.011 V vs. Ag/AgCl. This thermodynamically irreversible behavior with a large anodic-to-cathodic peak difference (ΔE) suggests slow electron-transfer kinetics. In the solution at pH 7.4, where the 4N form predominates, a significant decrease in the currents associated with the Cu(I)/Cu(II) conversion was observed, also the peak separation

Fig. 1. CV obtained for Cu(II) complexes with $A\beta(11-16)$ (A, B) and $pA\beta(11-16)$ (C, D) (1: 0.9 molar ratio) at pH 6.2 (black curves), 7.4 (red curves), and 9.0 (blue curves), recorded in 4 mM HNO_3 /96 mM KNO_3 , $\nu = 0.1$ V/s

decreased, $\Delta E = 0.280$, and the formal potential was shifted towards less positive potential values to $E_f = 0.096\text{V}$. (Fig. 1 C, D, magnified view in SI 3.7) When CV was registered at pH 9 no Cu(I)/Cu(II) was visible, as under those conditions only the 4N form exists (SI Table 3). The disappearance of the Cu(I)/Cu(II) signals resulted in an increase of the currents for the Cu(II)/Cu(III) process. The formal potential (E_f) of the Cu(II)/Cu(III) couple depends on the nitrogen atoms which bind Cu(II) ion. In the case of $\{\text{NH}_2, 3\text{N}-\}$ copper complexes E_f was estimated to $\sim 410\text{ mV}$ [26] but in the presence of histidine (His) in the peptide sequence (complex type $\{\text{Im}(\text{His}), 3\text{N}-\}$), E_f is shifted to more positive values: $\sim 660\text{ mV}$. [32] In our study the values of E_f obtained in a $\text{HNO}_3/\text{KNO}_3$ solution were $\sim 0.530\text{ V}$ and the process could be described as quasi-reversible (SI Table 2). As mentioned earlier, in comparison to $\text{A}\beta(11-16)$ (K_d value for pH 7.4 = $1,8\text{ pM}$) the pyroglutamate amyloid is a weaker Cu(II) ion chelator (K_d value for pH 7.4 = $5,8\text{ nM}$) which was reflected in the recorded voltammograms. The formal potential of the Cu(II)/Cu(III) process is around 0.2 V lower for $\text{pA}\beta(11-16)\text{-Cu(II)}$ than for $\text{A}\beta(11-16)\text{-Cu(II)}$. Registered DP voltammograms are in good agreement with CV. (SI 2.2, SI Table 2) At pH 6.2, two oxidation peaks related to the Cu(I)/Cu(II) process were observed which may be due to the presence of 2 stoichiometric forms of the 2N structure (see Table 3 SI)

Comparison of redox properties of Cu(II) coordinated by $\text{A}\beta(11-16)$ and $\text{pA}\beta(11-16)$ in phosphate buffer

Phosphate buffer, which better reflects conditions in the human body, is also very commonly used in electrochemical experiments as a supporting electrolyte. However, phosphate anions may interact with peptide complexes and change their properties. According to recent studies the $\text{A}\beta(5-x)$ family possess the His-2 motif and actuate the 3N binding mode where the fourth position can be occupied phosphate ions. [11] Tobolska et al. [12] reported that CV curves obtained for $\text{A}\beta(5-9)\text{-Cu(II)}$ complexes showed that phosphates preferentially affected the Cu(II)/Cu(III) couple, significantly decreasing the potential of Cu(II) oxidation whereas the influence on the Cu(II)/Cu(I) peak was minor. [12] ATCUN peptides with an XYH sequence compare to those with the XH (His-2) metal binding motif create with Cu(II) ions full saturated 4N structures. Despite this, Fraczyk, using spectroscopic techniques, showed that phosphorylation of serine residues markedly weakens the stability of ATCUN-Cu(II) complexes. [25] Up until now, there are few voltammetric studies where the influence of phosphoric ions on ATCUN complex properties are investigated. In one such case, Hureau et al. tested DAHK-Cu(II) in $0.05\text{ M Tris}/0.1\text{ M NaClO}_4$ and $0.1\text{ M phosphate buffer}$, both at pH 7.4 but no significant differences were observed. [32]

To compare the electrochemical behaviour of A β (11-16)-Cu(II) and pA β (11-16)-Cu(II) in different supporting electrolytes we performed experiments in 0.1 M phosphate buffer at pH 6.2, 7.4 and 9.0. The successive increase of pH caused the diffusion controlled Cu(II)/Cu(III) process for A β (11-16)-Cu(II) to take place at slightly lower potential compared to the KNO₃ (SI 2.1, 2.2) which indicates that this oxidation process is a little more favourable in the presence of phosphate ions. As previously, no peaks corresponding to Cu(I)/Cu(II) were detected on the 1st scan. (Fig. 2A). The same relationship was observed in the recorded DP voltammograms. (SI 2.2A). In cathodic potential ranges we observed the reduction Cu(II)/Cu(I) below -1 V. Interestingly, at pH 6.2 this process occurred at more a positive potential than at pH 7.4 and 9.0, which may suggest that the phosphate ions at lower pH will facilitate the process of re-organising the structure from square planar to linear. As expected on the 2nd scan the additional peak corresponding to the Cu(I)/Cu(II) oxidation was observed (Fig 2B).

A more significant effect of the phosphate ions on the electrochemical properties was observed for the weaker ligand copper(II) ions in pA β (11-16)-Cu(II) (Fig. 2C, D). At pH 6.2, both in the 1st and 2nd scans, only the Cu(I)/Cu(II) process at $E_f = 0.133$ V was observed, while in KNO₃ also the Cu(II)/Cu(III) couple was present. This is a further indication that the phosphate ions shift the reaction equilibrium towards Cu(I)/Cu(II) over Cu(II)/Cu(III) and could be connected with a preference to the 2N structure over the 4N. This low-potential reaction has been connected with the formation of harmful ROS under biologically relevant conditions.[4] It is also worth noting that the Cu(II)/Cu(III) reaction for pA β (11-16)-Cu(II) complex occurred at lower potential values than for A β (11-16)-Cu(II) in solutions of pH 7.4 and 9.0. (SI, Table 1,2). DP voltammograms are in excellent agreement with CV curves (SI 2.2.B).

Fig. 2. CV obtained for Cu(II) complexes with A β (11-16) (A, B) and pA β (11-16) (C, D) (1: 0.9 molar ratio) at pH 6.2 (black curves), 7.4 (red curves), and 9.0 (blue curves), recorded in 0.1 M phosphate buffer, $v = 0.1$ V/s

Conclusions

In the present work, we investigated well-soluble models of two N-terminal truncated β -amyloids which are the major component of senile plaques - extracellular neurotoxic structures considered as a hallmark of Alzheimer's Disease (AD). Voltammetric studies on the copper complexes of both A β (11-16) and its pyroglutamate counterpart, pA β (11-16), were performed to evaluate the influence on redox properties given by seemingly small changes in the peptide sequence. A β (11-16), which structure begins with glutamic acid, belongs to the ATCUN family and therefore forms a very stable 4N complex with Cu(II). This complex underwent both Cu(II)/Cu(III) oxidation and Cu(II)/Cu(I) reduction reactions, but anodic and cathodic processes could be recorded only at very high (above 0.7 V vs Ag/AgCl) and very low (below -1 V vs Ag/AgCl) potentials, what causes this complex to be redox silent in the human body. The cyclisation of glutamic acid to pyroglutamic acid removed the ATCUN type binding properties, resulting in lower affinity to Cu(II) ions and a strikingly different electrochemical response. Voltammetry (cyclic and differential pulse) of pA β (11-16)-Cu(II) complexes showed an additional electrochemical process, related to the Cu(I)/Cu(II) cycle, enabled by insufficient stabilisation of the Cu(II) redox state by pA β (11-16). Reactions at these potentials were previously observed for Cu(II)-A β (1-x) species [5-8] which means that conversion of A β (11-16) to pA β (11-16) results in a peptide that can contribute to the production of reactive oxygen species (ROS), compounds which damage nerve cells. In line with this, the Cu(II)/Cu(III) conversion was also registered at much lower potentials than for A β (11-16)-Cu(II) complexes. Additionally, the use of different supporting electrolytes (potassium nitrate and phosphate buffer) showed that in the case of pA β (11-16)-Cu(II) complexes, phosphate ions shift the

reaction equilibrium towards the process responsible for ROS formation. In conclusion, the electrochemical investigation of A β (11-16)-Cu(II) and pA β (11-16)-Cu(II) complexes revealed their distinct redox properties, which is essential for understanding the pathogenesis of Alzheimer's disease. Our study also underlines the importance of interactions with other species available in the human body, and the proper choice of electrolyte solutions.

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Conflicts of interest

There are no conflicts to declare

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