Amyloid Beta Peptide VHHQ, KLVFF, and IIGLMVGGVV Domains Involved in Fibrilization: AFM and Electrochemical Characterization

Teodor Adrian Enache, Ana-Maria Chiorcea-Paquim, and Ana Maria Oliveira-Brett*  
Department of Chemistry, Faculty of Sciences and Technology, University of Coimbra, 3004-535, Coimbra, Portugal

ABSTRACT: The time-dependent structural modifications and oxidation behavior of specifically chosen five short amyloid beta (Aβ) peptides, Aβ1−16, Aβ1−26, Aβ1−28, Aβ1−38, and Aβ1−42, fragments of the complete human Aβ1−40 peptide, were investigated by atomic force microscopy (AFM) and voltammetry. The objective was to determine the influence of different Aβ domains (VHHQ that contains electroactive histidine H residues, KLVFF that is the peptide hydrophobic aggregation core, and IIGLMVGGVV that is the C-terminus hydrophobic region), and of Aβ peptide hydrophobicity, in the fibrilization mechanism. The short Aβ peptides absence of aggregation or the time-dependent aggregation mechanisms, at room temperature, in free chloride media, within the time window from 0 to 48 h, were established by AFM via changes in their adsorption morphology, and by differential pulse voltammetry, via modifications of the amino acid residues oxidation peak currents. The first oxidation peak was of tyrosine Y residue and the second peak was of histidine H and methionine M residues oxidation. A correlation between the presence of an intact highly hydrophobic KLVFF aggregation core and the time-dependent changes on the Aβ peptides aggregation was found. The hydrophobic C-terminal domain IIGLMVGGVV, present in the Aβ1−40 peptide, also contributed to accelerate the formation of Aβ1−40 peptide aggregates and fibrils.

Each neuron of a normal healthy brain constantly exchanges information with others as electrical charges via neurotransmitters. Alzheimer’s disease (AD) compromises the capacity of neurons to communicate with each other, destroying over time memory and thinking skills.1−4 Amyloid beta plaques and neurofibrillary tangles are abnormal structures that are typical biological indicators of the AD.3,4

Synthetic amyloid beta (Aβ) peptide fragments, varying in length from 40 to 43 amino acids, were used for decades in Aβ aggregation studies, in the context of diseases, such as AD and neurodegeneration.5−7 It is a fact now that the aggregation process is a result of a complex series of oligomerization and polymerization events that follow a nucleation-dependent mechanism.8−11 While a connection between highly ordered fibril aggregates and neurodegeneration is maintained, there is evidence that fibrillar aggregates do not directly cause the neuronal death, but small soluble oligomers and fibrillar precursors of Aβ fibrils are the dominant neurotoxic species.6,8,12,13

The fibrils formed in solution are different from those formed at a solid surface, and the physicochemical properties of the surface affect the aggregation rate and aggregates shape.14,15 The fibrils formed in solution are long, continuous, twisted together into helices, and grow by attaching Aβ monomer units at the fibril end14,16,17 As more fragments are added, these structures increase in size and become insoluble, eventually forming Aβ plaques. At a solid surface, the fibrils can grow in any direction by adding building blocks composed by several monomers.14,18 Whether in solution or at a surface, from monomer to fully formed fibrils, each intermediate aggregate will potentially be unique in size, shape, and structure.1,2,15,19 However, the investigation of these intermediary structures is difficult due to the aggregation process that takes only several hours.20

Atomic force microscopy (AFM) and differential pulse (DP) voltammetry have been recognized as fruitful approaches to study the Aβ peptides structure and aggregation.14,15,20−26 Among the 20 amino acids present in peptides and proteins, only five are electroactive: tyrosine Y,27−29 tryptophan W,28,29 histidine H,30 methionine M,31 and cysteine C.31 Structurally, the Aβ peptides can contain only three electroactive amino acids residues: tyrosine Y, histidine H, and methionine M. The Aβ peptides voltammetric investigation showed that the electron transfer takes place at the electroactive tyrosine Y, histidine H,
and methionine M residues, Scheme 1.\textsuperscript{15,20,22,27,30–33} However, sometimes only the tyrosine Y\textsuperscript{10} oxidation was detected.\textsuperscript{20,22}

The electrochemical behavior of the $\alpha$-synuclein monomers, before the aggregation process started, was investigated.\textsuperscript{33} DP voltammetric results showed that the $\alpha$-syn 1–40 and $\alpha$-syn 1–42 peptides oxidation, at a glassy carbon electrode (GCE), occurs in two steps, the first electron transfer reaction corresponding to the tyrosine Y\textsuperscript{10} residue oxidation, and the second to the third histidine (H\textsuperscript{12}, H\textsuperscript{13}, and H\textsuperscript{14}) and one methionine (M\textsuperscript{55}) amino acid residues oxidation.\textsuperscript{33} The correct identification of the $\alpha$-syn 1–40 and $\alpha$-syn 1–42 peptides oxidation peaks was achieved before the fibrillation occurrence, in freshly prepared solutions, in free chloride media, based on the comparison between ten di(peptides in a fibrilization process.

The novelty of this paper consists in investigating, for the first time by electrochemistry and AFM, the fibrillation process from the point view of three distinct $\alpha$-syn peptide amino acid domains, Y\textsuperscript{12}HHQ\textsuperscript{15}, K\textsuperscript{16}LVFF\textsuperscript{20}, and I\textsuperscript{31}GLMVGGGV\textsuperscript{40}. This was achieved by AFM and voltammetric characterization of the structural modifications undertaken by specially chosen five short $\alpha$-syn sequences with different N-terminus, C-terminus, and amino acid lengths: $\alpha$-syn 1–16 $\alpha$-syn 1–28 $\alpha$-syn 10–20 $\alpha$-syn 12–28, and $\alpha$-syn 17–42, Scheme 1, in a time-dependent manner, within the time window from 0 to 48 h.

The aim was to understand how the central neighboring Y\textsuperscript{12}HHQ\textsuperscript{15} and K\textsuperscript{16}LVFF\textsuperscript{20} and C-terminus I\textsuperscript{31}GLMVGGGV\textsuperscript{40} amino acid domains, and the sequence hydrophobicity influenced the $\alpha$-syn peptides aggregation, and how the relationship between these domains affected the $\alpha$-syn peptides oxidative behavior.

The results obtained bring new information, complementary to other physical and chemical methods generally used to study the aggregation mechanisms (such as fluorescence microscopy, thioflavin T and Congo red assays), especially for understanding how the electroactive domain VHHQ oxidative behavior is affected by the close proximity to the nonelectroactive KLVFF domain, that is the $\alpha$-syn peptide hydrophobic aggregation core.

## EXPERIMENTAL SECTION

### Materials and Methods.

The $\alpha$-syn 1–16, $\alpha$-syn 1–28, $\alpha$-syn 10–20, $\alpha$-syn 12–28, $\alpha$-syn 17–42, and $\alpha$-syn 1–40 peptides were bought lyophilized from Sigma–Aldrich and Bachem and used without further purification.

Prior to all experiments, the $\alpha$-syn 1–16, $\alpha$-syn 1–28, $\alpha$-syn 10–20, $\alpha$-syn 12–28, $\alpha$-syn 17–42, and $\alpha$-syn 1–40 solutions were prepared according to the following procedure. The lyophilized powder was first dissolved in 1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg mL\textsuperscript{−1} to monomerize pre-existing aggregates. After that, the HFIP was evaporated under a vacuum pump, leaving a film that was redissolved in 0.1 M phosphate buffer pH 7.4. The suspension was further monomerized by sonication for 1 min, followed by dilution to 100 $\mu$M $\alpha$-syn peptide, in 0.1 M phosphate buffer pH 7.4 (stock solutions). The $\alpha$-syn peptide stock solutions were then kept at room temperature up to 48 h incubation time, in order to study the time-dependent absence/presence of aggregation. At different aggregation states (0, 24, and 48 h incubation time), aliquots of stock solutions were collected, diluted, and analyzed by AFM (dilution to 10 $\mu$M $\alpha$-syn peptide) and DP voltammetry (dilution to 50 $\mu$M $\alpha$-syn peptide).

All experiments were done at room temperature (25 ± 1 °C) in 0.1 M phosphate buffer pH 7.4, prepared with analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 $\mu$S cm\textsuperscript{−1}). Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co. Inc., Woburn, U.S.A.). The pH measurements were performed with a CRISON 2001 micro pH-meter with an InGold-combined glass electrode.

<table>
<thead>
<tr>
<th>Metal Binding Site</th>
<th>Functional Domain for Neurotoxic Effects</th>
<th>Highly Hydrophobic Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-syn 1–40</td>
<td>D'AEFRH'DGNY'EVH'H'QKLVFAEVDGSNKGAIGLM'H'GQGVV\textsuperscript{42}</td>
<td>$\alpha$-syn 1–10</td>
</tr>
<tr>
<td>$\alpha$-syn 1–16</td>
<td>D'AEFRH'DGNY'EVH'H'QKLVFAEVDGSNK\textsuperscript{28}</td>
<td>$\alpha$-syn 12–28, 17–42</td>
</tr>
<tr>
<td>$\alpha$-syn 1–28</td>
<td>D'AEFRH'DGNY'EVH'H'QKLVFAEVDGSNK\textsuperscript{28}</td>
<td>$\alpha$-syn 17–42</td>
</tr>
<tr>
<td>$\alpha$-syn 1–42</td>
<td>D'AEFRH'DGNY'EVH'H'QKLVFAEVDGSNK\textsuperscript{28}</td>
<td>$\alpha$-syn 17–42</td>
</tr>
</tbody>
</table>

"Electroactive amino acid residues: tyrosine Y, histidine H, and methionine M."
The amino acid residues in the Aβ peptides, Scheme 1, are described with one letter abbreviation according to the amino acids code used in the International Nucleotide Sequence Database: alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), serine (S), tyrosine (Y), tryptophan (W), and valine (V).

All the abbreviations used are listed in Table 1.

Table 1. List of Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Differential pulse</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>GCE</td>
<td>Glassy carbon electrode</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HOPG</td>
<td>Highly oriented pyrolytic graphite</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>R.S.D.</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>

**Atomic Force Microscopy.** AFM was performed in the acoustic AC mode, with a PicoScan controller and a CS AFM S scanner with a scan range of 6 mm in x–y and 2 μm in z (Agilent Technologies, U.S.A.). AppNano type FORT, 225 μm length, 3.0 N m⁻¹ spring constants, and 47–76 kHz resonant frequencies in air (Applied NanoStructures, Inc., U.S.A.) were used.

AFM images were topographical and were taken with 512 samples/line x 512 lines and scan rates of 0.8–2.5 lines s⁻¹. When necessary, the images were processed by flattening in order to remove the background slope and the contrast and brightness were adjusted.

Highly oriented pyrolytic graphite (HOPG) ZYB quality of 15 × 15 × 2 mm³ dimensions (Advanced Ceramics Co., U.S.A.) were used as substrate, because is atomically flat. GCE used for the voltammetric study is rough and therefore unsuitable for AFM surface characterization. Voltammetric experiments using HOPG and GCE showed similar electrochemical behavior. The HOPG was freshly cleaved with adhesive tape prior to each experiment and imaged by AFM.

For the AFM study, the Aβ₁₋₁₆, Aβ₁₋₂₈, Aβ₁₀₋₂₀, Aβ₁₂₋₂₈, Aβ₁₇₋₄₂, and Aβ₁₋₄₀ modified HOPG surfaces were prepared by depositing 50 μL of 10 μM Aβ peptide solution in 0.1 M phosphate buffer pH 7.4 onto the freshly cleaved HOPG, allowing the spontaneous adsorption over a period of 3 min. The excess of solution was then gently cleaned with a jet of Milli-Q water, and the Aβ-modified HOPG surfaces were dried in a sterile atmosphere and imaged by AFM in air.

Due to Aβ fibrils insolubility, Aβ peptides structural studies are difficult to perform, and methods such as single crystal X-ray crystallography and nuclear magnetic resonance (NMR) cannot be used. X-ray fiber diffraction, electron microscopy, solid state NMR, Fourier transform infrared spectroscopy and circular dichroism have been used to examine Aβ structure. However, Aβ/peptide unambiguous structural measurements are still under investigation. AFM has proved to be a valuable tool for investigating the nature of the intermediates in the Aβ peptide fibrillation pathway. The Aβ peptide morphological modifications observed by AFM were identified and correlated with the Aβ/peptide structural changes, based on the Aβ/peptide heights measured by AFM, and compared with other Aβ/peptide structural determinations.

**Differential Pulse Voltammetry.** Voltammetric experiments were carried out using an IVIUM potentiostat in combination with IviumSoft program version 2.219 (Ivium Technologies, Eindhoven, The Netherlands). Measurements were carried out using a GCE working (d = 1 mm), a Pt wire counter and an Ag/AgCl (3 M KCl) reference electrodes, in a one-compartment 2 mL electrochemical cell (Edaq, Europe). The experimental conditions for DP voltammetry were: pulse amplitude 50 mV and pulse width 100 ms at a scan rate of 5 mV s⁻¹.

Before each experiment, the GCE was polished using diamond spray (particle size 1 μm) on a microcloth pad, rinsed with Milli-Q water and electrochemically pretreated, by recording various DP voltammograms in buffer supporting electrolyte until a steady state baseline voltammogram was obtained.

For the voltammetric study, the Aβ₁₋₁₆, Aβ₁₋₂₈, Aβ₁₀₋₂₀, Aβ₁₂₋₂₈, Aβ₁₇₋₄₂, and Aβ₁₋₄₀ modified GCEs were prepared by depositing 50 μL of 50 μM Aβ peptide solution in 0.1 M phosphate buffer pH 7.4 onto the clean GCE, allowing the spontaneous adsorption over a period of 10 min. Then the GCE with adsorbed Aβ peptide was rinsed with Millipore Milli-Q water, placed in the electrochemical cell and DP voltammograms were recorded in 0.1 M phosphate buffer pH 7.4 supporting electrolyte.

The DP voltammograms were background-subtracted and baseline-corrected using the IviumSoft program tools. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artifact, although the peak height is in some cases reduced (<10%) relative to that of the untreated curve, and was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak currents obtained for each Aβ peptide sequence were lower than 8%.

**Results and Discussion.** Most Aβ peptide electrochemical studies are based on the detection of tyrosine Y oxidation peak, that difficult the
investigation of Aβ sequences not containing the tyrosine Y10 amino acid residue.

The research novelty consisted in the investigation by AFM and DP voltammetry, of three distinct Aβ peptide amino acid domains involved in fibrillation, which do not contain the tyrosine Y10 residue: (1) V12HHQ15 central domain that contains the electroactive histidine H13 and H14 residues, (2) K16LVFF20 domain that is the peptide hydrophobic aggregation core, and (3) I13GLMVGGVV40 domain that is the C-terminus hydrophobic region.

In order to understand the influence of these domains and of the Aβ peptide hydrophobicity, on the fibrillation mechanism, the redox behavior and structural modifications undergone by five, the human Aβ1−40 complete sequence, and four short synthetic fragments, Aβ1−16, Aβ1−28, Aβ10−20, Aβ12−28, and Aβ17−42, were investigated. The sequences were chosen in such a way to highlight the role played by the V12HHQ15, K16LVFF20,45,46 and I13GLMVGGVV40 domains, and the relationship between them, Scheme 1. The short Aβ1−16, Aβ1−28, Aβ10−20, Aβ12−28, and Aβ17−42 peptides absence of aggregation or the time-dependent aggregation mechanisms were established based on the correlations between the Aβ peptides redox behavior observed by DP voltammetry at a GCE, and conformational changes observed by AFM at a HOPG surface, and compared with the full length human Aβ1−40 peptide.

The Aβ peptide solutions were first incubated during time periods up to 48 h. Each Aβ peptide modified HOPG surface was prepared by 3 min spontaneous adsorption from 10 μM Aβ peptide solution, and imaged by AFM. Each Aβ peptide modified GCE was prepared by 10 min spontaneous adsorption from 50 μM Aβ peptide solution, and investigated by DP voltammetry in 0.1 phosphate buffer pH 7.4. During the aggregation and/or fibrilization, changes in the Y, H, and M amino acid residues oxidation currents are expected to occur.13 The short Aβ1−16, Aβ1−28, Aβ10−20, Aβ12−28, and Aβ17−42 peptides absence/presence of aggregation was electrochemically detected, by following the changes in the Aβ peptides oxidation peaks currents. Since the tyrosine Y10 residue was not present in any of the three domains investigated, special attention was given to the Aβ peptides second oxidation peak, corresponding to the histidine H and methionine M residues oxidation. The histidine H residues oxidation was especially important when studying the influence of the central domain V12HHQ15, which has never been studied before.

For the correct identification of the histidine H and methionine M oxidation peaks, the Aβ12−28 peptide that contains only the histidine H13 and H14, and the Aβ17−42 peptide that contains only the methionine M39 electroactive residues, Scheme 1, were used as control.

Aβ1−16 Peptide. The Aβ1−16 peptide, Scheme 1, has a molecular weight of 1955.01 Da, presents four electroactive amino acid residues, one tyrosine Y10 and three histidines H6, H13, and H14, being the most hydrophilic peptide studied, with a hydrophobicity of only 18.75%.

AFM images of the Aβ1−16 peptide at 0 h incubation showed a 1.20 ± 0.5 nm height network film with granular appearance, Figure 1A, corresponding to the adsorption of the short hydrophilic Aβ1−16 monomers. Increasing the incubation time to 24 and 48 h, Figure 1B, the Aβ1−16 Peptide film became progressively smoother, and the HOPG surface coverage increased. This is due to a reorganization of the Aβ1−16 monomer structure, from initially globular into predominantly α-helix conformations, without the formation of aggregates.

DP voltammograms recorded after 0 incubation, at the Aβ1−16 peptide modified GCE, showed the occurrence of two consecutive oxidation peaks, the first corresponding to the tyrosine Y10 residue oxidation, at Eo = +0.65 V, and the second one corresponding to the histidine H6, H13, and H14 residues oxidation, at Eo = +1.00 V, Figure 1C,D. Moreover, the oxidation peak currents remained constant when increasing the incubation time to 24 and 48 h, Figure 1D, showing that the Aβ1−16 peptide did not aggregate, in agreement and confirmed by AFM.

Aβ1−28 Peptide. The Aβ1−28 peptide, Scheme 1, has a molecular weight of 3262.46 Da, presents four electroactive amino acid residues, one tyrosine Y10 and three histidines H6, H13, and H14, and has a hydrophobicity of 32.14%.

AFM images of the Aβ1−28 peptide at 0 h incubation showed a smooth network film of 2.23 ± 0.2 nm height, Figure 2A. Increasing the incubation time to 24 and 48 h, Figure 2B, the HOPG surface coverage by the Aβ1−28 peptide decreased drastically. This phenomenon can be associated with the presence of the highly hydrophobic nonelectroactive K16LVFFA21 aggregation core, which, in solution, may lead to the formation of larger, more compact aggregates that do not adsorb onto HOPG.

DP voltammograms recorded after 0 h incubation, at the Aβ1−28 peptide modified GCE, showed the tyrosine Y10 residue oxidation peak, at Eo = +0.65 V, and the histidine H6, H13, and H14 residues oxidation peak, at Eo = +1.00 V, Figure 2C,D. Although the Aβ1−28 peptide presented the same electroactive residues and the same oxidation peaks as the Aβ1−16 peptide, Figure 1D, in the case of Aβ1−28 peptide much higher oxidation peak currents were recorded, Figure 2D. The larger redox waves were explained considering both the higher hydrophobicity (32.14% for Aβ1−28 comparative to 18.75% for Aβ1−16) and the greater adsorption of Aβ1−28 peptide on carbon surfaces, Figure 2A. However, increasing the incubation time, a slow decrease of the Aβ1−28 peptide oxidation peak currents occurred, Figure 1D.
Figure 2D, consistent with the time-dependent aggregation and adsorption decrease, Figure 2B.

**Aβ10−20 Peptide.** The Aβ10−20 peptide, Scheme 1, is the shortest Aβ peptide studied, with a molecular weight of 1446.65 Da, presents three electroactive amino acid residues, one tyrosine Y10 and two histidines H13 and H14, and has a hydrophobicity of 45.54%. AFM images of the Aβ10−20 peptide at 0 h incubation showed a network film with granular aspect, Figure 3 A, corresponding to the adsorption of Aβ10−20 monomers.

Increasing the incubation time to 24 and 48 h, Figure 3B, a slight aggregation was observed, leading to a reorganization of the Aβ10−20 peptides on the HOPG, and the formation of small clusters.

DP voltammograms recorded after 0 incubation, at the Aβ10−20 peptide modified GCE, showed two oxidation peaks, the tyrosine Y10 oxidation, at $E_p = +0.65$ V, and the histidine H13 and H14 oxidation, at $E_p = +1.00$ V, Figure 3C,D, whose currents decreased with increasing the incubation time, Figure 3D. The first oxidation peak, corresponding to the N-terminus tyrosine Y10 residue, decreased slowly from 0 to 24 h, and then the current remained constant, while the second oxidation peak, corresponding to the two histidine H13 and H14 residues, decreased faster and continuously. As in the case of Aβ12−28, Figure 2D, the Aβ10−20 peptide aggregation occurred and involved the hydrophobic nonelectroactive K16LVFFA21 aggregation core, which affected the availability to oxidation of the two neighbor histidine H13 and H14 residues, but did not influence the accessibility to oxidation of the tyrosine Y10, the first residue in the Aβ10−20 peptide sequence.

**Aβ12−28 Peptide.** The Aβ12−28 peptide, Scheme 1, has a molecular weight of 1955.18 Da, presents only two electroactive histidine, H13 and H14 amino acid residues, and has a hydrophobicity of 41.18%.

AFM images of the Aβ12−28 peptide at 0 h incubation showed a smooth network film, Figure 4A, similar to the film formed by the Aβ12−28 peptide, Figure 2A, but with a lower degree of surface coverage, due to its shorter length. The HOPG coverage by Aβ12−28 peptides decreased with increasing the incubation time to 24 and 48 h, Figure 4B, and only a small number of spherical aggregates up to 10 nm of height were observed.

The Aβ12−28 peptide hydrophobicity of 41.18% is between the hydrophobicity of the Aβ1−28 (32.14%) and the Aβ10−20 peptide (45.54%). The DP voltammograms, recorded after 0 h incubation, at the Aβ12−28 peptide modified GCE, showed only.
one oxidation peak corresponding to the two histidine H13 and H14 residues, at $E_p = +1.00$ V, Figure 4C,D, with an oxidation peak current comparable with that of the $\alpha$- and $\beta$-peptides, Figure 2D, and $\gamma$-secretase. As observed for $\alpha$- and $\beta$-peptides, $\alpha$-peptides also presented a slow decrease of the oxidation peak currents with increasing the incubation time, Figure 4D, caused by the aggregation induced by the highly hydrophobic non-electroactive K16LVFFA21 aggregation core.

The $\alpha$- and $\beta$-peptides were used as controls for the identification of the $\alpha$- and $\beta$-peptides. Considering that the $\alpha$- and $\beta$-peptides contain only the histidine H13 and H14 electroactive residues, Scheme 1, the oxidation peak observed undoubtedly corresponds to the histidine H oxidation.

$\alpha$- 17– 42 Peptide. The $\alpha$- 17–42 peptide, Scheme 1, has a molecular weight of 2577.05 Da, contains only the electroactive methionine M35 amino acid residue, being the most hydrophobic sequence, with a hydrophobicity of 61.54%. In addition, the $\alpha$- 17–42 peptide is the only sequence studied that did not contain the electroactive V12HHQ15 domain.

The $\alpha$- 17–42 peptide is very important to study the effect of the I31IGLMVGGVVIA42 C-terminus hydrophobic domain, known to accelerate the fibrilization of $\alpha$- over $\beta$-peptide. In fact, the $\alpha$- 17–42 peptide is not actually a fragment of the $\beta$-peptide, but a longer fragment of the transmembrane amyloid peptide, but a longer fragment of the transmembrane amyloid precursor protein (APP), generated after the proteolytic cleavage of the proteases $\beta$- and $\gamma$-secretase.

AFM images of the $\alpha$- 17–42 peptide after 0 h incubation showed a very low HOPG coverage, with only a few globular aggregates of 1.72 ± 0.2 nm height, Figure 5A. The $\alpha$- 17–42 peptide was the only sequence that presented such a low HOPG surface coverage at 0 h incubation.

Due to its unique structure with two hydrophobic regions at both the N- and C-terminus, the $\alpha$- 17–42 peptide quickly underwent morphological changes, forming aggregates with the hydrophobic nonelectroactive domains L17VFFA21 and I13VFFA21 buried from water inside the $\alpha$- 17–42 peptide.7 The adsorption pattern remained unchanged with increasing the incubation time to 24 and 48 h, Figure 5B.

DP voltammograms recorded at 0 incubation, at the $\alpha$- 17–42 peptide modified GCE, showed only one small oxidation peak, corresponding to the methionine M35 residue oxidation, at $E_p = +1.00$ V, Figure 5C,D, which remained constant with increasing the incubation time to 24 and 48 h, Figure 5D.

The $\alpha$- 17–42 peptide is also used as control in $\alpha$- and $\beta$-peptide amino acid residues electrochemical identification. Considering that $\alpha$- 17–42 peptide contains only the methionine M35 electroactive residue, Scheme 1, the oxidation peak observed undoubtedly corresponds to the methionine M35 oxidation to methionine sulfoxide.

The methionine M35 residue very low oxidation peak current, while compared with other electroactive amino acid residues, is related with both the $\alpha$- 17–42 peptide low adsorption at the carbon surface, Figure 5A,B, and the formation of $\alpha$- 17–42 compact aggregates with the methionine M35 residue hidden inside the aggregates and unavailable to reach the GCE for oxidation.

$\beta$ 14–40 Peptide. The human $\beta$ 14–40 peptide, Scheme 1, has a molecular weight of 4329.80 Da, presents five electroactive amino acid residues, one tyrosine Y10, three histidines H6, H13, and H14, and one methionine M35, and a hydrophobicity of 42.50%. The $\beta$ 14–40 peptide is present in nearly 80% of the plaques formed in AD brain.

In chloride-free media and at room temperature, the $\beta$ 14–40 peptide underwent morphological changes, from soluble, monomeric random coil or $\alpha$-helix conformations, into aggregated $\beta$-sheet structures, a fibrillation process that involved different intermediate structures, including the formation of globular oligomers and protofibrils.15

In freshly prepared solutions, at 0 h incubation, the $\beta$ 14–40 peptide was in a soluble, monomeric, random coil or $\alpha$-helix conformation.15 AFM images of $\beta$ 14–40 peptide after 0 h incubation showed a densely packed film of 1.95 ± 0.4 nm height, Figure 6A, corresponding to the adsorption of the hydrophilic...
$\beta_{1-40}$ monomers. As expected, at 0 h incubation, the longer $\beta_{1-40}$ monomers covered to a great extent the HOPG, Figure 6A, when compared with the shorter sequences: $\beta_{1-16}$, Figure 1A, $\beta_{1-28}$, Figure 2A, $\beta_{10-20}$, Figure 3A, and $\beta_{12-28}$, Figure 4A. However, in the case of the $\beta_{1-40}$ peptide, the hydrophobic HOPG surface induced rapid changes from monomer into $\beta$-sheet configurations, and the $\beta_{1-40}$ peptides adsorbed as spherical aggregates, Figure 6A, white arrows, and short protofibrils of 2.5–6.0 nm height, Figure 6A, red arrows. 15

As the $\beta_{1-40}$ fibrilization progressed, more changes in the adsorption morphology occurred. 15 After 24 h incubation, the number of soluble $\beta_{1-40}$ monomers in solution drastically decreased, which resulted in a decrease of the adsorbed random-coiled structures and an increase on the number of the spherical aggregates with heterogeneous height values, varying between 2.0 and 7.0 nm. After 48 h incubation, Figure 6B, the HOPG coverage by $\beta_{1-40}$ peptides was very low. No random-coiled $\beta_{1-40}$ monomers were detected, the number of the aggregates decreased, and several long, smooth, branched fibrils could be observed, Figure 6B, green arrow. 15

DP voltammograms recorded, at 0 h incubation, at the $\beta_{1-40}$ peptide modified GCE showed the occurrence of two consecutive oxidation peaks, Figure 6C,D. The first oxidation corresponded to the tyrosine Y16 residue, at $E_p = +0.65$ V, while the second oxidation peak, at $E_p = 1.00$ V, was due to the three histidine, H8, H13, and H14, and one methionine M35 residues. Although the $\beta_{1-40}$ peptide is the only sequence that presents all five electroactive amino acid residues, both oxidation peak currents were significantly lower than the oxidation peak currents of smaller peptides: $\beta_{1-16}$ (lacks M35), Figure 1D, $\beta_{1-28}$ (lacks M35), Figure 2D, $\beta_{10-20}$ (lacks H8 and M35), Figure 3D, and $\beta_{12-28}$ (lacks H8, Y10, and M35), Figure 4D. The low oxidation peak currents observed by DP voltammetry, corroborated with the adsorption of small oligomers and protofibrils observed by AFM, Figure 6A, showing that at 0 h incubation, the $\beta_{1-40}$ peptide nucleation process already started. The $\beta_{17-42}$ peptide was the only sequence that presented a lower M35 oxidation peak current, Figure 5D, since it lacked the contribution of the three histidine residues, H8, H13, and H14, and the $\beta_{17-42}$ peptide sequence also started the aggregation process at 0 h incubation.

The time-dependent $\beta_{1-40}$ peptide fibrilization process was electrochemically detected via the two oxidation peak currents decrease, with increasing the incubation time from 0 to 24 and 48 h, Figure 6D. After 48 h incubation, the faradic signal of the DP voltammogram was merging with the background current, due to the disappearance of all $\beta_{1-40}$ monomers, Figure 6B, now converted into aggregates and/or fibrils with $\beta$-sheet conformations that presented the electroactive amino acid residues protected inside the aggregates and not easy to be oxidized at the GCE.

The short $\beta_{1-28}$, $\beta_{10-20}$, $\beta_{12-28}$, and human $\beta_{1-40}$ peptides are the only sequences that presented an intact highly hydrophobic nonselectroactive K16LVFF domain aggregation core, being also the only ones that showed a time-dependent aggregation. The fact that the short $\beta_{1-28}$, $\beta_{10-20}$, $\beta_{12-28}$ peptides aggregated, although they lack the hydrophobic sequence K16LVFF domain, confirmed the critical role played by the K16LVFF domain in the aggregation process.

The $\beta_{17-42}$ Peptide presented only a truncated aggregation core L17VFF domain, and showed a time-independent aggregation. The $\beta_{17-42}$ and $\beta_{1-40}$ peptides were the only sequences that started the aggregation process immediately in the freshly prepared solution, at 0 h incubation, while the short $\beta_{1-28}$ $\beta_{10-20}$ and $\beta_{12-28}$ peptides, which lacked the C-terminus hydrophobic sequence I13GLMVGGV domain, only started the aggregation after a longer incubation time. This confirmed the ability of the $\beta$ peptides hydrophobic C-terminus I13GLMVGGV domain to regulate the rate of $\beta$ fibril formation, more than the stability and the structural properties of the $\beta$ peptide. 34,48

### CONCLUSIONS

The results brought new information concerning the reactivity of the H13 and H14, amino acid residues and the oxidative behavior of the electroactive domain VHHQ, influenced by the close proximity to the hydrophobic KLVFF aggregation core. The short $\beta_{1-16}$, $\beta_{10-20}$, $\beta_{12-28}$ and human $\beta_{1-40}$ peptides time-dependent aggregation and fibrilization, induced by the presence of the intact nonelectroactive domain KLVFF, the peptide hydrophobic aggregation core, was detected. AFM images showed a decrease in the adsorption and/or the occurrence of aggregates protofibrils and fibrils that was correlated with the decrease in the oxidation peak currents, observed by DP voltammetry. The hydrophobic C-terminal domain I13GLMVGGV is present in the long $\beta_{1-40}$ Peptide also contributed to accelerate its aggregation rate.

The short $\beta_{1-16}$ peptide was the most hydrophilic of the peptides studied, and the only one that did not undergo aggregation with increasing the incubation time, due to the lack of the hydrophobic KLVFF aggregation core, and of the C-terminal domain I13GLMVGGV.

The $\beta_{17-42}$ peptide was longer, did not contain the electroactive VHHQ domain and also had a truncated LVFF aggregation core. However, due to the presence of two hydrophobic regions at both the N- and C-terminus, the $\beta_{17-42}$ peptide formed aggregates immediately in freshly prepared solution, that did not change with increasing the incubation time.

### AUTHOR INFORMATION

**Corresponding Author**

Tel./Fax: +351-239-835295. E-mail: brett@ci.uc.pt.

**ORCID**

Ana Maria Oliveira-Brett: 0000-0002-6244-0891

**Notes**

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

Financial support from Fundação para a Ciência e Tecnologia (FCT), Grant SFRH/BPD/92726/2013 (A.-M.C.-P.), UID/EMS/00285/2013 (cofinanced by the European Community Fund FEDER), FEDER funds through the program COMPETE - Programa Operacional Factores de Competitividade, and SUDOE project Innovec/EAU (SOE1/P1/F0173) are gratefully acknowledged.

### REFERENCES


(9) Blak, G.-B.; Choe, Y.-J.; Paik, S.-R. RMB Rep. 2009, 42 (9), 541–551.
DOI: 10.1021/acs.analchem.7b04686
Anal. Chem. 2018, 90, 2283–2292