Synthesis of Different Analogs of $A\beta_{(9-16)}$ Peptide
Mass spectrometric evidence for heavy metal binding

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Amyloid-β (Aβ) peptides are proteins associated with Alzheimer's disease (AD), because the extracellular Aβ deposits are the main cause of this disorder. The aggregation of Aβ has been shown to depend on the interactions with metal ions, such as copper, zinc, aluminum or iron. The N-terminal sequence of $A\beta_{(1-42)}$ or $A\beta_{(1-40)}$ peptides, namely $A\beta_{(1-16)}$ peptide fragment, is considered the metal binding site involved in AD neurodegeneration and amyloidogenesis. Therefore, we have investigated different peptide sequences to understand the role played by some amino acid residues in metal binding. In this paper, we report the chemical synthesis of $A\beta_{(9-16)}$ peptide and its analogs by Fmoc/tBu strategy and the mass spectrometric evidence for metal ion binding to newly synthesized peptides. MALDI-ToF mass spectrometry proved to be a reliable tool to detect and identify the metal ion complexes of all peptides investigated with copper, iron and zinc ions.

Keywords: Chemical synthesis, amyloid peptides, heavy metal binding, copper, iron, zinc, mass spectrometry, Alzheimer's disease

Numerous metal ions are thought to be involved in the production of reactive oxygen species (ROS), which is the case of metal binding to amyloid-β (Aβ) peptides associated with Alzheimer's disease (AD) [1]. Oxidative stress is an important aspect of this pathology and is one of the first signs of Alzheimer's disease. However, its exact role of ROS in AD is not known, it still unclear what is the initial source of oxidation stress in AD, and a supposition says it could be related to redox-active metals such as copper or iron [2, 3]. Is known that numerous effects of oxidative stress causes the appearance of ROS [4]. These are responsible for the oxidation of proteins, lipids and DNA in the brain, leading to cell damage and blockage to their functions [5]. Howerer, studies using oxidised polysaccharides as crosslinking agents improved the formation of bioactive stroma tissue [6]. Research in this field shows that oxidative stress is an event that anticipate the appearance of the pathologies of this disorder such as, neurofibrillary tangles and amyloid plaques [7-9]. However, there are several approaches to prevent the Aβ peptide aggregation. Some of them use neuropeptides such as NAP or SAL that play an important role in neuroprotection and immunity [10-13]. Furthermore, the protective role of those peptides against metal toxicity has been highlighted in different studies [14]. Following interaction of Aβ oligomers with Fe^{3+} or Cu^{2+} ions, the reduction of metal ions is facilitated along with reactive ROS and hydrogen peroxide (H_2O_2) production [4, 15].

The synthesis of peptides and their metal complexes is a highly researched domain mainly due to conformational changes that may occur in the structure of peptides, these mechanisms are being responsible for the appearance of conformational diseases that are not fully elucidated [16-19].

Therefore, this work aims at presenting the synthesis of the following new peptides: H_2N-GFEVHHQK- $CONH_2$ ($A\beta_{(9-16)}F$), H_2N-GGEVHHQK-$CONH_2$ ($A\beta_{(9-16)}G$), H_2N-GYEVGGQK-$CONH_2$ ($A\beta_{(9-16)}GG$) and H_2N-GGEVGGQK-$CONH_2$ ($A\beta_{(9-16)}GGG$), respectively, which are analogs of H_2N-GYEVHHQK-$CONH_2$ ($A\beta_{(9-16)}$). In addition, we studied the affinity of all these peptides toward copper, iron and zinc ions.

Experimental part

Materials and methods

All chemicals and solvents were of analytical grade and purchased from commercial sources. The solutions were prepared using ultrapure water from a Milli-Q system (Millipore, Bedford, MA). Amino acids used for synthesis were protected at the α-amide group with Fmoc (9-fluorenylmethyloxycarbonyl) and were purchased from GL Biochem (Shanghai). As solid support for the peptide synthesis was used a rink amide resin procured from Sigma Aldrich. Dimethylformamide (DMF) was obtained from Carl Roth GmbH (Karlsruhe, Germany), while dichloromethane (DCM) and diethyl ether were purchased from Scharlab S.L. (Barcelona, Spain). Piperidine (PYP), 4-methyl-morpholine (NMM), triisopropylsilan (TIS), trifluoroacetic acid (TFA), bromophenol blue, ethanol, acetonitrile (ACN) and the activator PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) were achieved from Merck (Germany). Metal salts were purchased from Merck, whereas α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma-Aldrich (Germany).

Instruments. Sample weighing was done with the analytical balance Vibra HT (Japan). Incubation at 24°C it was carried out using an Eppendorf compact thermomixer purchased from Germany. For lyophilization of the samples, was used a lyophyliser from CHRIST ALPHA 1-2 LDplus. MALDI-ToF MS analysis was carried out with a Bruker Ultraflex MALDI ToF/ToF mass spectrometer operated in positive reflectron mode and equipped with a pulsed

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nitrogen UV laser (λmax 337 nm). The obtained spectra were processed using Bruker´s Flex Analysis 3.4 software.

Mass spectra. For MALDI-ToF MS analysis, the samples were co-crystallized with an excess of organic matrix capable to absorb at 337 nm and volatilize under the action of laser radiation. As matrix was used a saturated solution of α-cyano-4-hydroxy-cinnamic acid (HCCA) dissolved in a solution containing 2: 1 ACN: 0.1% TFA in MilliQ was used for peptide mapping. Using the dried drop method, that implies adding first 1 µL of sample and over it 1 µL of freshly prepared matrix solution, the mixture was deposited on a conductive metallic plate called target and allowed to dry. After co-crystallization, the metal plate was introduced into the mass spectrometer and bombarded with short laser pulses. The desorbed and ionized molecules were accelerated by an electrostatic field and discharged through a high-fly metal flight tube. Depending on their mass, ionized molecules reach the detector at different times.

The spectra were recorded in positive reflectron mode using the following parameters: 20 kV acceleration voltage, 40% grid voltage, 140 ns delay, low mass gate of 500 Da and an acquisition mass range of 600-3500 Da. The final mass spectrum represents an accumulation of 300 shots per acquisition.

Peptide synthesis. The following five peptides (100 micromoles) were synthesized: H_2N-GYEVHHQK-$CONH_2$ ($A\beta_{(9-16)}$), H_2N-GFEVHHQK-$CONH_2$ ($A\beta_{(9-16)}F$), H_2N-GGEVHHQK-$CONH_2$ ($A\beta_{(9-16)}G$), H_2N-GYEVGGQK-$CONH_2$ ($A\beta_{(9-16)}GG$) and H_2N-GGEVGGQK-$CONH_2$ ($A\beta_{(9-16)}GGG$), respectively. Peptide synthesis was performed manually from the C-terminus of the peptide to the N-terminus by the Fmoc/tBut Solid Phase Synthesis (SPPS) method as previously described [20, 21].

Preparation of metal complexes: The newly synthesized peptides $A\beta_{(9-16)}$, $Av_{(9-16)}F$, $A\beta_{(9-16)}G$, $Av_{(9-16)}GG$ and $A\beta_{(9-16)}GGG$ were dissolved in deionized water to obtain a 4 mM stock solution. The metal was added in a 1:10 peptide to metal molar ratio. The resulted solutions were incubated in a thermomixer (Thermomixer Compact Eppendorf AG 22331, Germany) for 20 h at 24° and 350 rpm.

Results and discussions

A method that ensures rapid determination of molecular weight is MALDI-ToF mass spectrometry [22]. The structure of the newly synthesized peptides was confirmed by determining their molecular weight with a MALDI-ToF mass spectrometer. Table 1 shows the general structure of the synthesized amyloid peptide $A\beta_{(9-16)}$ and its analogs. By comparing the theoretical m/z values obtained using the ChemCalc software with the m/z values obtained from the MALDI-ToF mass spectrometer it was possible to confirm the complex formation between the fragment $A\beta_{(9-16)}$, derived from the amyloid peptide, and ions of copper, iron and zinc, as shown in Table 2.

Table 1

GENERAL STRUCTURE OF THE NEWLY SYNTHESIZED PEPTIDES, ANALOGS OF $A\beta_{(9-16)}$ PEPTIDE

Peptide Code	*Sequence*	*Structure*
$A\beta_{(9-16)}$	GYEVHHQK	H_2N-GYEVHHQK-NH_2
$A\beta_{(9-16)}F$	GFEVHHQK	H_2N-GFEVHHQK-NH_2
$A\beta_{(9-16)}G$	GGEVHHQK	H_2N-GGEVHHQK-NH_2
$A\beta_{(9-16)}GG$	GYEVGGQK	H_2N-GYEVGGQK-NH_2
$A\beta_{(9-16)}GGG$	GGEVGGQK	H_2N-GGEVGGQK-NH_2

Figure 1 shows the MS spectrum of $A\beta_{(9-16)}$ peptide and its analogs in the presence of Cu(II) ions. In all cases, the reduction of Cu(II) ions to Cu(I) prior to complex formation with Aβ peptides was noted. In all experiments, no signal corresponding to the peptide complex containing Cu(II) was found in the mass spectra. According to the values theoretically calculated in the case of native peptide $A\beta_{(9-16)}$ the signal for the molecular ion $[M+H]^+$ should be at m/z 996.47 and the complex $[M+Cu(I)]^+$ at m/z 1058.62. A peak characteristic to the molecular ion $[M+H]^+$ at m/z 996.4 was identified, whereas the characteristic signal for the complex with copper $[M+Cu(I)]^+$ appeared at m/z

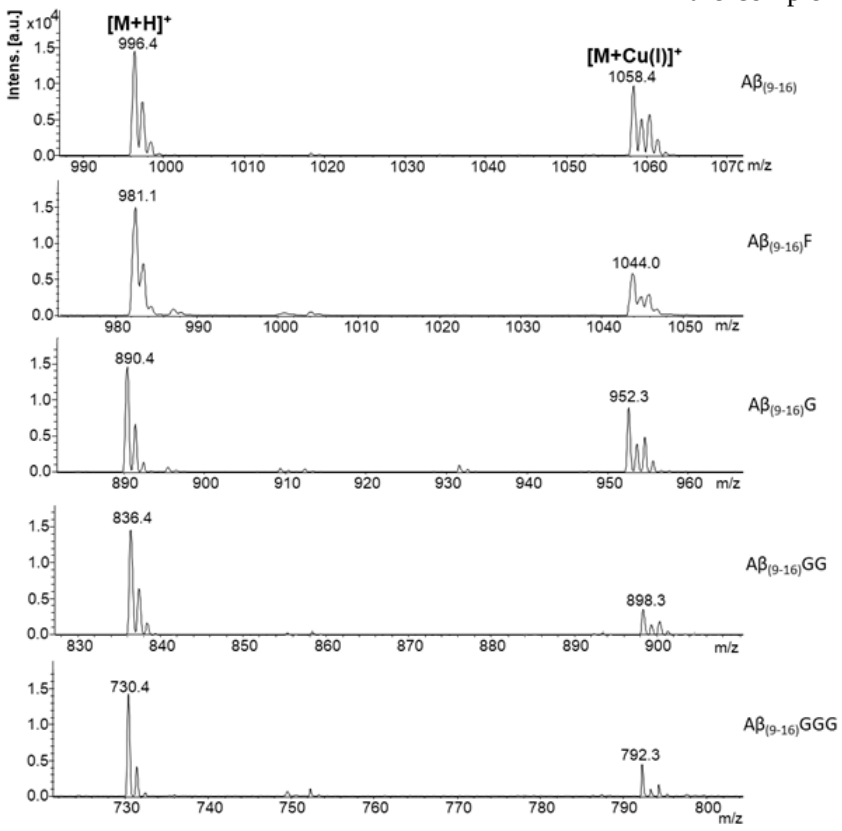

Fig. 1. MALDI-ToF MS spectra of $A\beta_{(9-16)}$ and its analogs in the presence of copper ions at a 1:10 peptide to metal ratio

1058.4. For the peptide modified with phenylalanine $A\beta_{(9-16)}F$, the theoretically calculated value for the molecular ion $[M+H]^+$ was found at m/z 981.09, while that for the complex with copper ion $[M+Cu(I)]^+$ at m/z 1043.62. In fact, the signal for the molecular ion $[M+H]^+$ was identified at m/z 981.1 and the signal for the complex with copper $[M+Cu(I)]^+$ appeared at m/z 1044.0. In addition, the signal for the molecular ion $[M+H]^+$ of $A\beta_{(9-16)}G$ (the peptide modified with glycine), was observed at m/z 890.45, whereas that of the complex $[M+Cu(I)]^+$ at m/z 952.38. Practically, two signals, one for the molecular ion $[M+H]^+$ at m/z 890.4 and another one for the complex with Cu(I) ions $[M+Cu(I)]^+$ at m/z 952.3 were noticed. The glycine enriched peptides $A\beta_{(9-16)}GG$ and $A\beta_{(9-16)}GGG$, according to the theoretically values, gave the signal for the molecular ion $[M+H]^+$ at m/z 836.4 and that of the complex with copper $[M+Cu(I)]^+$, at m/z 898.3, in the case of $A\beta_{(9-16)}GG$. As for the peptide Aβ(9-16)GGG the molecular ion $[M+H]^+$ was found at m/z 730.4 and the complex $[M+Cu(I)]^+$ at m/z 792.3. In all cases, no signal corresponding to the peptide complex: Cu(II) was found in the mass spectrum. In addition, although MALDI ToF does not quantitate the concentrations of metal-peptide complexes, the affinity for metal ion apparently decreased with the numbers of glycine found in the peptide structure. More exactly, the richest glycine peptide $A\beta_{(9-16)}GGG$, displayed the lowest signal for the $[M+Cu(I)]^+$ complex.

Although we used iron(III) salts, Figure 2 displays the mass spectra of $A\beta_{(9-16)}$ peptide and its analogs, which shows complexes with iron (II) ions. As for the native peptide $A\beta_{(9-16)}$, the values practically obtained confirmed the theoretically calculated values. Thus, we found the molecular ion $[M+H]^+$ at m/z 996.47 and the complex $[M+Fe(II)-H]^+$ at m/z 1050.42. From the registered MS spectrum, we identified the signal for the molecular ion $[M+H]^+$ at m/z 996.4, whereas the peak for the complex with iron $[M+Fe(II)-H]^+$ appeared at m/z 1050.5. The peptide modified with phenylalanine $A\beta_{(9-16)}F$ showed two peaks, one corresponding to the molecular ion $[M+H]^+$ at m/z 981.1 and another one for the complex with iron $[M+Fe(II)-H]^+$ at m/z 1035.5. With respect to $Ab_{(9-16)}F$, the molecular ion $[M+H]^+$ was noticed at m/z 981.09, and the complex with iron $[M+Fe(II)-H]^+$ at m/z 1035.43. The peptide modified with glycine, $A\beta_{(9-16)}G$, had two peaks, one corresponding to the molecular ion $[M+H]^+$ at m/z 890.4, and another one for the complex with iron $[M+Fe(II)-H]^+$ at m/z 944.4. In this case the theoretical values were very close to those obtained, namely at m/z 890.45 characteristic to the molecular ion $[M+H]^+$, and at m/z 944.38 for the complex with iron $[M+Fe(II)-H]^+$. The last two peptides, those rich in glycine residues, $A\beta_{(9-16)}GG$ and $A\beta_{(9-16)}GGG$, displayed the molecular ion $[M+H]^+$ at m/z 836.4 for $A\beta_{(9-16)}GG$ and at m/z 730.4 for $A\beta_{(9-16)}GGG$ in the mass spectra. These values are in good agreement with the theoretical ones both for the molecular ion $[M+H]^+$, present at m/z 836.91 for $A\beta_{(9-16)}GG$ and at m/z 730.79 for $A\beta_{(9-16)}GGG$. The complex with Fe(II) was also formed as it can be noticed from the spectra, but the signals had low intensity relative to those of peptides. Thus, the peptide $A\beta_{(9-16)}GG$ formed a complex with iron $[M+Fe(II)-H]^+$ found at m/z 890.5, while the complex of $A\beta_{(9-16)}GGG$ was present at m/z 784.3.

Figure 3 refers to the mass spectra of native $A\beta_{(9-16)}$ and its analogs in the presence of zinc (II) ions. In the mass spectrum of native $A\beta_{(9-16)}$, a signal for the molecular ion $[M+H]^+$ at m/z 996.4 can be observed, while the complex with zinc $[M+Zn(II)-H]^+$ was seen at m/z 1059.6. The experimental values were close to the theoretical ones, so that the molecular ion $[M+H]^+$ was shown at m/z 996.47, and the complex with zinc $[M+Zn(II)-H]^+$ at m/z 1059.47. As for the peptide $A\beta_{(9-16)}F$, there were two signals in the spectrum, one characteristic to the molecular ion $[M+H]^+$ at m/z 981.1 and another one, of low intensity, was identified at m/z 1044.5 of the complex with zinc $[M+Zn(II)-H]^+$. The theoretical values were m/z 981.09 for the molecular ion $[M+H]^+$ and m/z 1044.48 for the complex with zinc $[M+Zn(II)-H]^+$. The peptide $A\beta_{(9-16)}G$, modified with glycine, had also two peaks, one identified at m/z 890.4 equivalent to the molecular ion $[M+H]^+$ and another

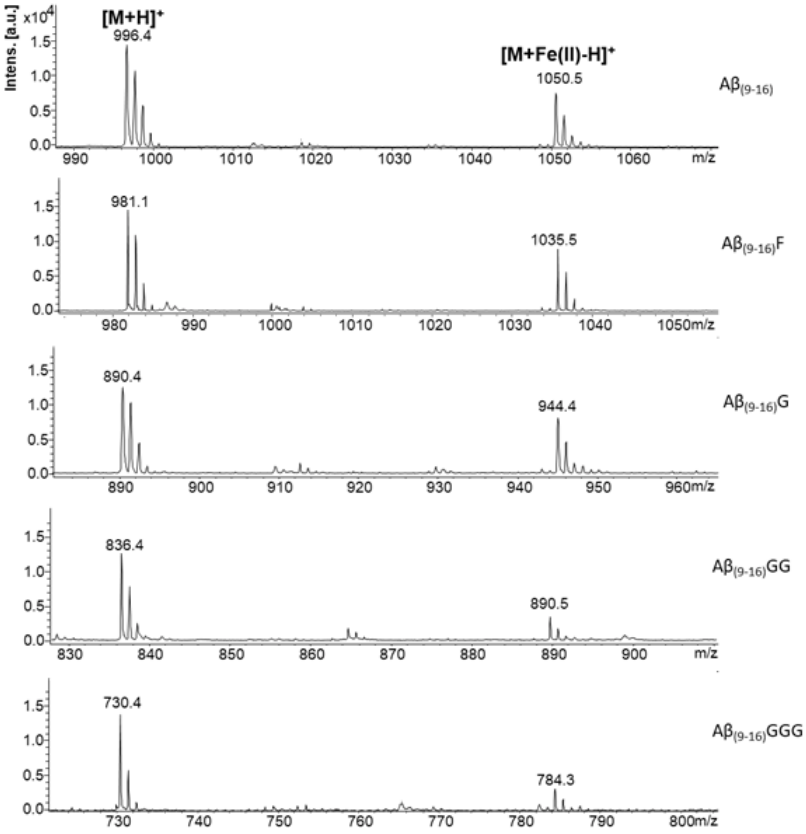

Fig. 2. MALDI-ToF MS spectra of Aâ$_{(9-16)}$ and its analogs in the presence of iron(III) ions, which are reduced to iron(II) before binding to peptides, at a 1:10 peptide to metal ratio

one, of low intensity, at m/z 954.4 that corresponds to the complex with zinc $[M+Zn(II)-H]^+$. According to the theoretically calculated values, the signal for the molecular ion $[M+H]^+$ was found at m/z 890,45 and the complex $[M+Zn(II)-H]^+$ at m/z 954.36. For the last two glycine enriched peptides, $A\beta_{(9-16)}GG$ and $A\beta_{(9-16)}GGG$, no complex formation was observed in the mass spectra. We identified

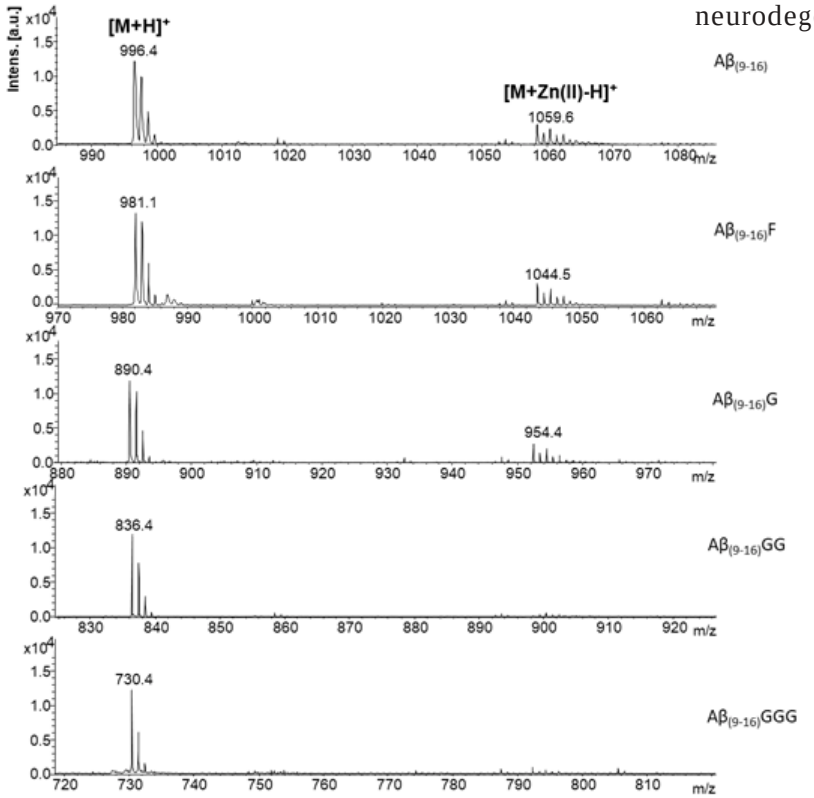

Fig. 3. MALDI-ToF MS spectra of $A\beta_{(9-16)}$ and its analogs in the presence of zinc ions at a 1:10 peptide to metal ratio

the peaks for the molecular ions $[M+H]^+$ at m/z 836.4 characteristic for $A\beta_{(9-16)}GG$ and at m/z 730.4 for $A\beta_{(9-16)}GGG$. Such values correspond to the theoretically calculated molecular weights. This experiment suggested that this type of glycine modified peptide lost its metal bonding properties.

Our results are in line with the main research on peptide synthesis of amyloid-b fragments associated with neurodegeneration. AD is an effective impairment of neurons and their functions, being generally manifested by neuronal and synaptic loss and a continuous degradation of cognitive activity [23, 24]. The most important hallmarks of Alzheimer are the deposit of amyloid plaques and neurofibrillar tangles in the brain [25-27]. This type of neurodegeneration is among the most studied and common neurodegenerative disorders [28-31]. Neuron death and the loss of cognitive functions is believed to be linked directly with brain atrophy, in which the brain of AD pacients can suffer a considerable reduction of the volume as compared to healthy people [1, 32-34].

The major component of the amyloid plaques is a small peptide called Aβ, which was first isolated by Glenner and Wong in 1984 [35]. This peptide is produced by proteolytic cleavage of the amyloid precursor protein (APP) [36]. Aβ has a molecular mass around 4.3 kDa and consists of 38-

Peptide	*Molecular Ion*	*Theoretical (m/z)*	*Experimental (m/z)*
$A\beta_{(9-16)}$	$[M+H]^+$	996.47	996.4
(H_2N-GYEVHHQK-$CONH_2$)	$[M+Cu(I)]^+$	1058.62	1058.4
$C_{44}H_{65}N_{15}O_{12}$	$[M+Fe(II)-H]^+$	1050.42	1050.5
	$[M+Zn(II)-H]^+$	1059.47	1059.6
$A\beta_{(9-16)}F$	$[M+H]^+$	981.09	981.1
(H_2N-GFEVHHQK-$CONH_2$)	$[M+Cu(I)]^+$	1043.62	1044.0
$C_{44}H_{65}N_{15}O_{11}$	$[M+Fe(II)-H]^+$	1035.43	1035.5
	$[M+Zn(II)-H]^+$	1044.48	1044.5
$A\beta_{(9-16)}G$	$[M+H]^+$	890.45	890.4
(H_2N-GGEVHHQK-$CONH_2$)	$[M+Cu(I)]^+$	952.38	952.3
$C_{37}H_{59}N_{15}O_{11}$	$[M+Fe(II)-H]^+$	944.38	944.4
	$[M+Zn(II)-H]^+$	954.36	954.4
$A\beta_{(9-16)}GG$	$[M+H]^+$	836.91	836.4
(H_2N-GYEVGGQK-$CONH_2$)	$[M+Cu(I)]^+$	898.89	898.3
$C_{36}H_{57}N_{11}O_{12}$	$[M+Fe(II)-H]^+$	890.34	890.5
	$[M+Zn(II)-H]^+$	900.30	-
$A\beta_{(9-16)}GGG$	$[M+H]^+$	730.79	730.4
(H_2N-GGEVGGQK-$CONH_2$)	$[M+Cu(I)]^+$	792.30	792.3
$C_{29}H_{51}N_{11}O_{11}$	$[M+Fe(II)-H]^+$	784.30	784.3
	$[M+Zn(II)-H]^+$	794.18	-

Table 2
MOLECULAR WEIGHT OF THE NEWLY SYNTHESIZED PEPTIDES, EXPERIMENTALLY DETERMINED WITH A MALDI-ToF INSTRUMENT AND CALCULATED WITH ChemCalc SOFTWARE

43 amino acid residues and is found in the amyloid plaques in the majority form having 42 amino acids residues [37]. Even if the main species of Aβ produced by neuronal cells is Aβ having 40 amino acids residues it was noted that the peptide Aβ quite longer appears to be important in the etiology of the disease. Anyway, Aβ was found in a soluble form into healthy brain and in an aggregated form in AD patient brain [1, 38].

The presence of metals in amyloid plaques has long been attributed to a connection between the formation of amyloid fibrils and metal ions [39, 40]. There are a number of different mechanisms through metal ions can aggravate AD [41]. By the interaction of metal ions with peptides, the conformation of peptide suffers some modifications with the formation of peptide-metal complexes with or without aggregation and peptide fibril formation [42].

Among the most important aspects of AD are the interaction of Aâ peptide with metals, the aggregation of Aβ, or the ability to induce oxidative stress [43]. One important aspect, we have focused in this paper, is the '*metal hypothesis* which refers to the interaction of Aβ peptide with different metal ions, such as copper, iron and zinc. According to this, metal ions are binding to Aβ and may produce misfolding and plaque aggregation of this peptide. One important aspect is we can detect the changes by mass spectrometry (MS) [44].

Therefore, we have synthesized four new eight amino acid residue-peptide fragments derived from the $A\beta_{(9-16)}$ peptide and investigated by MALDI-ToF MS copper, zinc and iron binding under physiological conditions. We noticed especially copper and iron reduction before binding to peptides, which suggest the role played by amyloid-β peptides in the process of ROS formation.

Conclusions

In this paper, we synthesized by solid phase peptide synthesis, Fmoc/tBu strategy, four new Aβ peptides, analogs of $A\beta_{(9-16)}$ peptide, namely H_2N-GFEVHHQK-$CONH_2$, H_2N-GGEVHHQK-$CONH_2$, H_2N-GYEVGGQK-$CONH_2$, and H_2N-GGEVGGQK-$CONH_2$, followed by the investigation of their interaction with metal ions, such as copper, iron and zinc by MALDI ToF mass spectrometry. Thus, it was possible to notice, in all cases, the reduction of Fe^{3+} and Cu^{2+} ions to Fe^{2+} and Cu^{+} followed by their binding to the synthesized $A\beta_{(9-16)}$ peptides. All the peptides had low affinity toward zinc ions, but the glycine-containing ones do not bind zinc.

In addition, although MALDI-ToF does not quantitate the concentrations of metal-peptide complexes, the affinity for metal ion apparently decreased with the numbers of glycine found in the peptide structure. More exactly, the richest glycine peptide $A\beta_{(9-16)}$GGG, displayed the lowest signal for the metal ions complex.

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