Interaction of β-amyloid(1-40) peptide with pairs of metal ions: An electrospray ion trap mass spectrometric model study

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ABSTRACT

The stoichiometries and the affinity toward simple and paired metal ions of synthetic amyloid-β(1-40) peptide (Aβ1-40) were investigated by electrospray ion trap mass spectrometry (ESI-MS), circular dichroism (CD), and atomic force microscopy (AFM). The results lead to the working hypothesis that pH-dependent metal binding to Aβ1-40 may induce conformational changes, which affect the affinity toward other metals. A significant copper and zinc binding to Aβ1-40 peptide at pH 5.5 was found, whereas nickel ions commonly bind to each molecule of β-amyloid peptide. Some complexes of Aβ1-40 with more than one nickel ion were identified by ESI-MS. In addition, nickel ions proved to enhance Aβ oligomerization. On increasing pH, up to 12 ions of zinc may bind to a single Aβ molecule. Under the same pH and concentration conditions, the binding pattern of the independent copper and silver ions to Aβ1-40 was different from that of the equimolecular mixture of the two metal ions. One might assume that some conformational changes due to water loss altered the capacity of Aβ peptide to bind certain heavy metal ions. As a consequence, copper–silver interaction with the binding process to Aβ1-40 became highly complex. A competition between silver and nickel ions for Aβ1-40 binding sites at high pH was also observed. New strategies were proposed to identify the characteristic signals for some important metal ion–peptide complexes in the spectra recorded at high pH or high concentrations of metal ions. To explain the formation of such a large number of high metal ion–Aβ complexes, we took into consideration the participation of both histidine residues and free amino groups as well as carboxylate ones in the binding process. Finally, CD and AFM studies supported the mass spectrometric data.

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1. Introduction

Electrospray-ionization mass spectrometry (ESI-MS) and circular dichroism (CD) spectroscopic investigations have confirmed that some sites of β-amyloid peptide (Aβ) are able to specifically bind metal ions [1,2]. Metal ions may interact with both amyloid precursor protein (APP) and Aβ to potentiate Alzheimer's disease by participating in the aggregation of these normal cellular proteins and in the generation of reactive oxygen species [3–5]. These metals are normally found at high concentrations in the regions of the brain most susceptible to AD neurodegeneration. Lowering Cu concentrations can downregulate the transcription of APP, showing that APP and Aβ form part of the Cu homeostatic machinery in the brain [6]. Aβ peptide, which is over accumulated in the AD brain, can efficiently generate reactive oxygen species in the presence of the transition metals [7]. In fact, increased brain metal levels have been associated with normal aging and a variety of diseases, including Alzheimer's disease (AD) [8]. Aβ has anti-oxidant properties, and at low levels, it has trophic rather than toxic properties and these protective effects have been attributed to the antioxidant properties of metallated Aβ [9]. The β-amyloid plaques that are one of the pathological features of Alzheimer's disease are composed of amyloid-β peptides and are highly enriched in metal ions such as Zn, Fe and Cu [10,11]. High concentrations of Zn^{2+} and Cu^{2+} are released during neurotransmission, which might explain why Aβ precipitation into amyloid commences in the synapse [12,13]. Contrary, the addition of low levels of zinc induces protease-resistant aggregation and precipitation of synthetic Aβ [14]. Besides, zinc ions afford protection against Aβ toxicity at low concentrations and enhance toxicity at high concentrations [15]. Copper and iron also induce peptide aggregation that is increased at acidic pH [16]. The involvement of metal ions as a potential key factor towards conformational modifications and aggregation of amyloid is widely recognized [17]. Circular dichroism (CD) studies indicated quite minimal ordering of the main-chain with Zn^{2+} or Cd^{2+} addition and that Zn^{2+} coordination is distinct from that of Cu^{2+} ions [18]. Copper,

iron, and zinc bind Aβ, generate an allosterically ordered membrane-penetrating oligomer, induce aggregation and give rise to reactive oxygen species [19]. Iron(II) or iron(III) blocks Cu-mediated neuro-toxicity in a highly reducing environment [20]; lipophilic ferrous chelators are effective agents for moderating neuronal damage in conditions such as AD where intracellular oxidative stress plays a significant role in disease pathology [21].

Nevertheless, less is known how toxic and essential metals may influence each other in regard to their retention by the peptides. We considered that a basic treatment of Aβ peptide containing two heavy metals (copper(II) and silver(I) or silver and nickel(II)) might reveal some interactions which take place between these metal ions and proteins within the living bodies under various pH conditions. Therefore, the aim of the present study is to detail the stoichiometries and binding properties of multiple cooperative metal ion-binding sites on synthetic Aβ1-40. By the introduction of additional methodological strategies the obtained spectra were simplified and some extra information was obtained. It is also our intention to shed some light on the relationship between various metal ions and amyloid peptide, as well as its impact on AD pathology.

2. Experimental

2.1. Reagents

Triethanolamine, methyl alcohol, silver nitrate, nickel chloride, ferric chloride, zinc chloride, copper sulfate, and mercury chloride were purchased from Merck (Darmstadt, Germany). All stock solutions of metal ions were prepared using MilliQ grade water (18 MΩ).

2.2. Synthesis of β-amyloid(1-40) peptide

Peptide H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-NH$_2$ (Aβ1-40) was synthesized manually, by SPPS, according to the Boc/Bzl strategy using MBHA resin (0.5 mmol/g coupling capacity) as a support. The following Boc-protected amino acid derivatives were used: Boc-Val-OH, Boc-Gly-OH, Boc-Met-OH, Boc-Leu-OH, Boc-Ile-OH, Boc-Ala-OH, Boc-Lys(ClZ)-OH, Boc-Asn-OH, Boc-Ser(Bzl)-OH, Boc-Asp(OcHex)-OH, Boc-Glu(OcHex)-OH, Boc-Phe-OH, Boc-Gln-OH, Boc-His(Bom)-OH, Boc-Tyr(BrZ)-OH and Boc-Arg(Tos)-OH.

The protocol of the synthesis was as follows: 1) DCM washing (3×0.5 min); 2) Boc cleavage using 33% TFA in DCM (2+20 min) for the first 5 cleavage steps and 33% TFA, 3% anisole in DCM containing 50 mg L-methionine and 10 mg DTT/10 mL cleavage solution (2+20 min) in order to avoid methionine oxidation; 3) DCM washing (5×0.5 min); 4) neutralization with 10% DIEA in DCM (4×1 min); 5) DCM washing (4×0.5 min); 6) coupling 3 or 5 equiv of Boc-amino acid derivative-DCC–HOBt in DCM–DMF mixture (60 min); 7) DMF washing (2×0.5 min); and 8) DCM washing (2×0.5 min). Monitor the coupling steps by ninhydrin assay to assess the presence of free amines. In the case of negative results a new cycle was started. The cycle was repeated from the neutralization step when the colorimetric test indicated ineffective coupling. After assembling the peptide chain on the resin, the N-terminal Boc-protecting group was removed prior to the final cleavage with HF.

2.3. Deprotection and cleavage of the peptide from the resin with HF

After removal of the N-terminal Boc-protecting group, the peptide-resin was neutralized and washed with DCM (4 times) and ethanol (2 times, 0.5 min). Before HF cleavage the resin was dried in a desiccator overnight. The cleavage of the side chain protecting groups and the detachment of the peptide from the resin by HF was performed in a Teflon apparatus (Peptide Institute, Osaka, Japan). For 1 g of peptide-resin 10 mL of liquid HF was used in the presence of 1 g p-cresol, 0.1 g DTT and 0.1 g L-cys. The cleavage was carried out for 90 min at 0 °C. After evaporation of HF (captured in a CaO-filled tower) the peptide was precipitated with cooled diethyl ether. The mixture of precipitated peptide and resin was filtered and washed 3–4 times with diethyl ether (30–40 mL) to remove scavengers. The unprotected peptide was dissolved in 10% acetic acid solution and then lyophilized.

2.4. Reverse phase high performance liquid chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Knauer system (H. Knauer, Bad Homburg, Germany) using an analytical Phenomenex Jupiter C$_4$ column (250×4.6 mm I.D.) with 5 µm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 55 min 100% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20 v/v)) was used at a flow rate of 1 mL/min at ambient temperature. Peaks were detected at 220 nm.

2.5. ESI ion trap mass spectrometry

Electrospray ionization mass spectrometric experiments and tandem mass spectrometry were carried out on a Bruker Daltonics Esquire 3000Plus (Bremen, Germany) ion trap mass spectrometer. Spectra were acquired in the 200–2000 m/z range. Samples were dissolved in a methanol/water 1:1 (v/v) solvent mixture, containing 0.1% acetic acid, and the pH was maintained at 5.5 with acetic acid or triethanolamine. Binding of individual metal ions to β-amyloid peptides was studied by ESI ion trap MS at pH 5.5, at a peptide concentration of 10 pmol/µL and 1:10 peptide:metal ion ratio. The competition of various metal ions towards the binding sites of Aβ peptide was investigated at a 1:5:5 molar ratio (Aβ1-40 peptide:metal 1:metal 2), pH 5.5, Aβ1-40 concentration being 10 pmol/µL. In order to study the binding of the individual metal ions to peptide at higher pH, triethanolamine was added instead of acetic acid to obtain pH 8.00, 9.00, and 9.20, respectively. Besides, the binding of metal ions to Aβ1-40 was studied at pH 7.4 or 6.6 using 5 mM ammonium acetate as a solvent. All masses were calculated using the charge state ruler tools provided with Bruker Daltonics DataAnalysis software of MS instrument. This software is able to select all of the various charged molecular ions, corresponding to the same peptide or metal–peptide complex.

In order to identify the characteristic signals for some important metal ion–peptide complexes in more complicated spectra we applied two main strategies. First, the spectra of the complexes with low metal ion–peptide ratios were carried out. Next, ESI-mass spectra of higher metal ion–peptide ratio complexes or those at high pH were recorded. The signals found in the first spectra were identified in the more complicated spectra, using also the charge state ruler.

2.6. Circular dichroism (CD) spectroscopy

CD spectra were recorded using a Jasco spectropolarimeter model J-710 (Labor and Datentechnik GmbH, Germany). All measurements were performed in quartz cells with a path length of 0.5 mm. Data were collected at the wavelengths from 180 to 260 nm in 0.2 nm increments. Four individual measurements were averaged in order to obtain the reported CD spectrum. The measurements were carried out at 25.0 ± 0.2 °C. Calculations of the fractional percentage of secondary structures were carried out using the computer programs Spectra-Manager (Jasco), CD-Deconvolution and Dicroprot [22].

2.7. Atomic force microscopy (AFM)

Images were taken at room temperature (22 °C) on a SPM Solver PRO-M AFM (NT-MTD Co. Zelenograd, Moscow, Russia) using the tapping mode. All images were acquired using a high resolution no contact "Golden" silicon NSG10/Au/50 cantilever with Au conductive coating. The cantilever was 100 µm in length, 35 µm in width, and with a 2 µm thickness and had a typical tip radius of 10 nm. A

resonant frequency of 254 kHz and a force constant of 11.5 N/m were applied. All AFM images were obtained at a resolution of 256×256 pixels on a scale of 10 μm × 10 μm. Image analysis was done with scanning probe microscopy software, WSxM 4.0 Develop 10.0 (Nanotec Electronica S. L.).

3. Results

Synthetic Aβ1-40 peptide showed characteristic peaks at m/z 722.3 ($[M + 6H]^{6+}$, the highest peak), and 866.5 for $[Aβ + 5H]^{5+}$. The molecular ions $[M–H_2O + 6H]^{6+}$ and $[M–H_2O + 5H]^{5+}$ also appeared in the spectrum and displayed relative intense signals at m/z 719.4 and 863.0, respectively (Fig. 1,a). In addition, the aspartic acid-lacking Aβ1-40, namely y39 fragment, with MW 4211.1 was identified, being more visible in the spectra of metal ion–peptide complexes.

3.1. Copper binding to Aβ1-40 at pH 5.5

Our study revealed a significant copper binding to Aβ1-40 peptide at pH 5.5 and a 1:1 Cu:Aβ molar ratio (Fig. 1,b). The Cu–Aβ complex appeared in the spectrum at m/z 628.1 (7+), 732.5 (6+), 878.8 (5+), and 1098.3 (4+), respectively. The second copper atom was present in the following copper–peptide ions: $[M + 2Cu + 3H]^{7+}$ (m/z 636.8), $[M + 2Cu + 2H]^{6+}$ (m/z 742.9), $[M + 2Cu + H]^{5+}$ (m/z 891.0), and $[M + 2Cu]^{4+}$ (m/z 113.5).

No complex between two or more molecules of peptide and copper ions was identified in the mass spectrum at acidic pH.

The relative intensity of $[M + Cu + 4H]^{6+}$ was 83.8% as compared to that of $[M + 6H]^{6+}$, whereas the corresponding ion $[M–H_2O + Cu + 4H]^{6+}$ was only 27.7% of the $[M–H_2O + 4H]^{6+}$ intensity. These data showed that possible conformational changes due to water loss diminished the capacity of the amino terminal area of Aβ peptide to bind certain heavy metal ions.

3.2. Zinc binding to Aβ1-40

At pH 5.5, Aβ1-40 molecules may bind up to two zinc ions with different intensities. Whereas the seven charged ions of the peptide did not appear in the mass spectrum, the zinc–Aβ peptide complex showed an intense signal at m/z 628.3 (Fig. 1c), which was assigned to $[M + Zn + 5H]^{7+}$ ion. Aβ–Zn complex was also confirmed by the peaks at m/z 732.8 (6+), 879.0 (5+; a less intensive signal), and 549.9 (3.5% of the intensity of 722.3 peak, $[M + Zn + 6H]^{8+}$).

The Zn–Aβ complex with Zn:Aβ molar ratio 2:1 appeared in the spectrum at m/z 743.3 (17.8%, $[M + 2Zn + 2H]^{6+}$), 637.8 (7.6%, $[M + 2Zn + 3H]^{7+}$), and 892.2, (4.2% $[M + 2Zn + H]^{5+}$), respectively.

The loss of a molecule of water reduced the zinc binding capacity of Aβ1-40 probably due to conformational changes. Nevertheless, a small peak at m/z 751.9 was noted, which was assigned to $[M–H_2O + 3Zn]^{6+}$.

3.3. Nickel binding to Aβ1-40

Aβ1-40 peptide displayed a high affinity toward nickel ions even at low pH (Fig. 1,d). Up to three nickel ions bound to each molecule of β-amyloid peptide as demonstrated by the peaks at m/z 722.2, 731.7, 741.2, and 750.6, which were assigned to $[M + 6H]^{6+}$, $[M + Ni + 4H]^{6+}$, $[M + 2Ni + 2H]^{6+}$, and $[M + 3Ni]^{6+}$, respectively. On the other hand, the signals of five-charged ions at m/z 877.8 and 888.9 clearly confirmed the formation of Ni–Aβ1-40 complexes in the molar ratios 1:1 and 1:2. Low intensity signals, which were assigned to the complex of Aβ1-40 with four nickel ions, were also found at m/z 912.4 (5+), 760.6 (6+), 652.1 (7+), 570.7 (8+), 507.2 (9+), 380.8 (12+), and 304.9 (15+), respectively.

3.4. Silver and iron binding to Aβ1-40

Both silver and iron ions displayed different binding patterns as compared to the other investigated metal ions. The intense signal at m/z 740.1 was assigned to Ag–Aβ1-40 complex (Fig. 1,e). Water loss from the Aβ molecule may result in conformational changes with lower metal binding capacity, which was evident from both Aβ complexes with silver and the other metal complexes (Fig. 1,b–e). The other signal for Ag–Aβ1-40 complex at m/z 887.8 with lower intensity as compared to the $[M + Ag + 5H]^{6+}$ signal was assigned to $[M + Ag + 4H]^{5+}$ (Fig. 1,e).

Iron(III) ion produced both the oligomerization of the peptide and iron-rich high molecular mass complexes. Besides the above mentioned signals, iron ions produced many other Fe–Aβ species that were identified with the help of the charge state ruler (Fig. 2). Because the main signals of Fe–Aβ complexes hindered the display of other lower signals, we had to investigate a narrower m/z range, close to m/z 722.3 (m/z of $[M + 6H]^{6+}$). Here, 5+, 6+, and 7+ ions of Aβ1-40 together with the multi-charged ions of multi-ratio Fe-Aβ1-40 complexes, including that of $[M + 3Fe-3H]^{6+}$ ion, were found in the spectrum. In fact, a large group of Fe(III)–Aβ1-40 complexes was identified with the help of a single ESI ion trap mass spectrum, starting from that of the Aβ1-40 pentamer with only one iron ion, $[5M + Fe + 32H]^{35+}$, and ending to the complex with nine iron ions bound to one Aβ1-40 molecule. Although iron binding to peptides will be discussed elsewhere, the presence of high molecular complexes such as that containing four molecules of Aβ and six iron ions should be outlined.

3.5. Metal–metal interactions during the binding process

The previous results demonstrated that potential target sites for the evaluation of metal binding need to be carefully analyzed for interfering metal–metal interactions [19,23–28]. In this study, decreases of bound silver by Aβ, and an increase in bound copper were the most pronounced interactions between the two toxic metals (Fig. 3). Under the same pH and concentration conditions, the binding pattern of the independent copper and silver ions to Aβ1-40 was different from that of the equimolecular mixture of the two metal ions. First, Aβ1-40 bound easily one copper ion to form the $[M + Cu + 4H]^{6+}$ complex, which was detected at m/z 732.5. Only a small part of Aβ1-40 molecules produced complexes containing two copper ions bound to one Aβ molecule (m/z 742.7; $[M + 2Cu + 2H]^{6+}$). Water-lacking Aβ1-40 had a lower affinity towards copper ions than the normal Aβ1-40 (Fig. 3,a; m/z 729.7). Second, both Aβ1-40 and Aβ1-40–H2O molecules proved to have a high affinity toward silver ions (Fig. 3,b), forming mono-silver complexes (m/z 737.3 and 740.1, respectively). The bi-silver complexes at m/z 757.9 corresponding to $[M + 2Ag + 4H]^{6+}$ were not considered because of their low intensity.

The picture of copper–silver interaction to Aβ1-40 was highly complex and had a completely unexpected pattern (Fig. 3,c). In addition to the peaks in the individual spectra of copper and silver, characteristic signals for peptide oligomerization by copper ions (m/z 738.3; $[2M + 3Cu]^{6+}$) and those for ternary complexes ($[M + Ag + Na + 4H]^{6+}$, m/z 744.0; $[M + Cu + Ag + 3H]^{6+}$, m/z 749.3) were found (Table 1). Apparently, copper diminished the signal of $[M + Ag + 5H]^{6+}$ ion at m/z 740.1, but stimulated the formation of silver complexes with water-loss Aβ1-40 from m/z 737.3. For example, $[M–H_2O + Ag + 5H]^{6+}$ and $[M–H_2O + 2Ag + 4H]^{6+}$ displayed intense signals at m/z 737.3 and 755.5, respectively. Silver ions potentated the binding capacity of copper ions to Aβ, including Aβ1-40 without one molecule of water. Thus, the intense signals at m/z 763.6 and 761.7 were assigned to $[M + 4Cu-2H]^{6+}$ and $[M–H_2O + 4Cu-2H]^{6+}$. We hypothesized that silver binding to Aβ1-40 peptide and especially to Aβ1-40–H2O one might result in the formation of silver–Aβ complexes in such a conformation that would enable them to react easily with copper ions. In this case, silver ions probably catalyze copper binding

to Aβ peptides via a reaction mechanism based on conformational changes and replacing peptide-bound silver ions by copper ions.

Within these experiments, the charge state ruler proved to be quite effective in identifying the peaks in the complicated spectra of non-covalent complexes of peptides.

3.6. Metal ion binding to Aβ1-40 at basic pH

Relatively small pH changes may affect the metal ion-binding capacity of the peptides and proteins. On increasing the pH due to the use of triethanolamine, the solution containing Aβ peptide may provide rather complicated mass spectra in the presence of individual metal ions. The high pH ESI-MS spectra of proteins or even simple peptides are far too complex for interpretation as most of the signals overlap heavily. Under the same conditions, the spectra of metal ion–peptide adducts become rather complicated but not impossible to be deciphered. By the introduction of additional methodological strategies, such as the comparison of complicated spectra with those obtained at low pH and lower metal ion concentration, as well as the use of the charge state ruler the
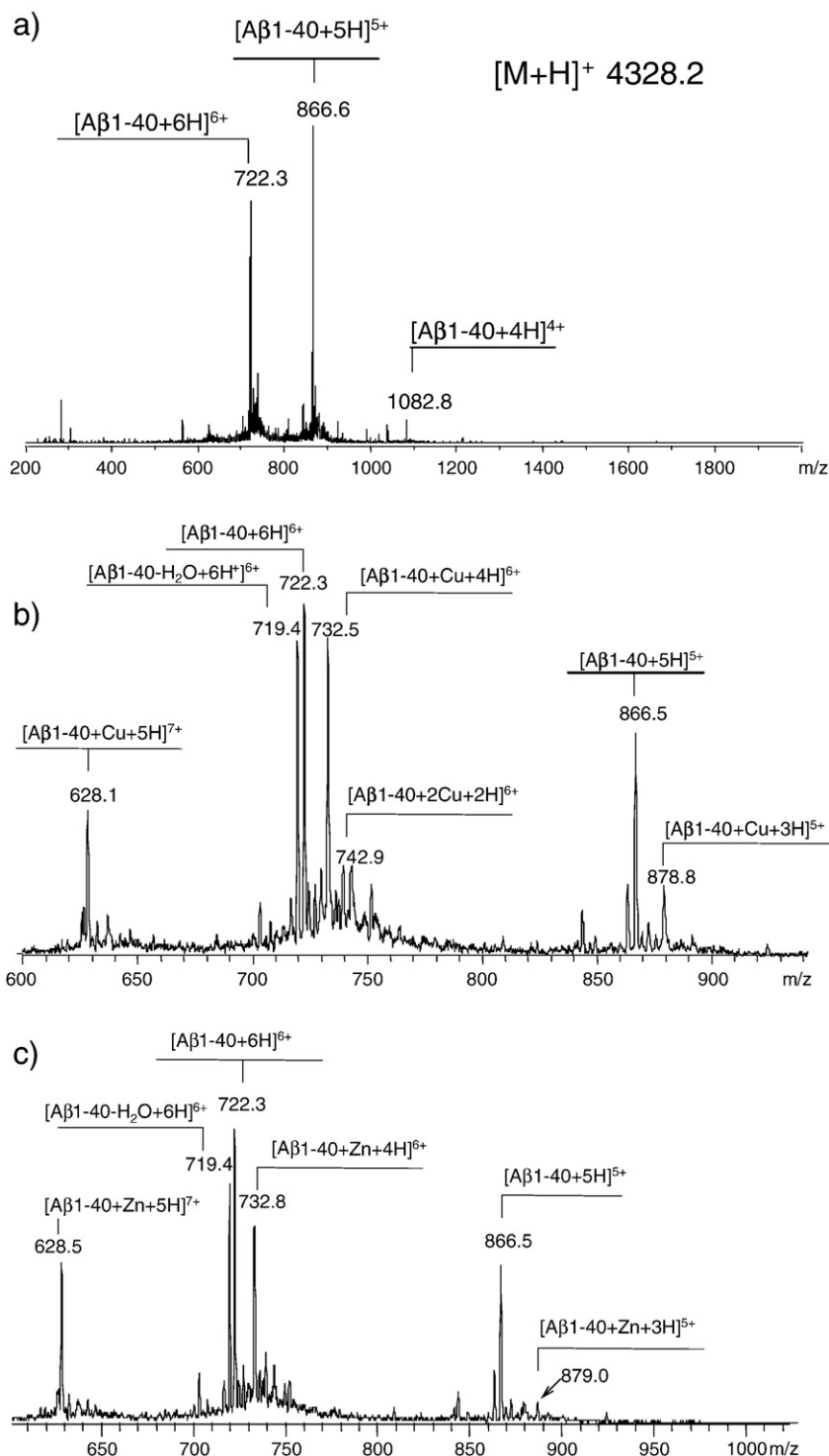

Fig. 1. ESI ion trap mass spectrum of Aβ1-40 (a) as well as its complexes with copper(II) [49] (b), zinc(II) (c), nickel(II) (d), and silver(I) (e) ions at pH 5.5.
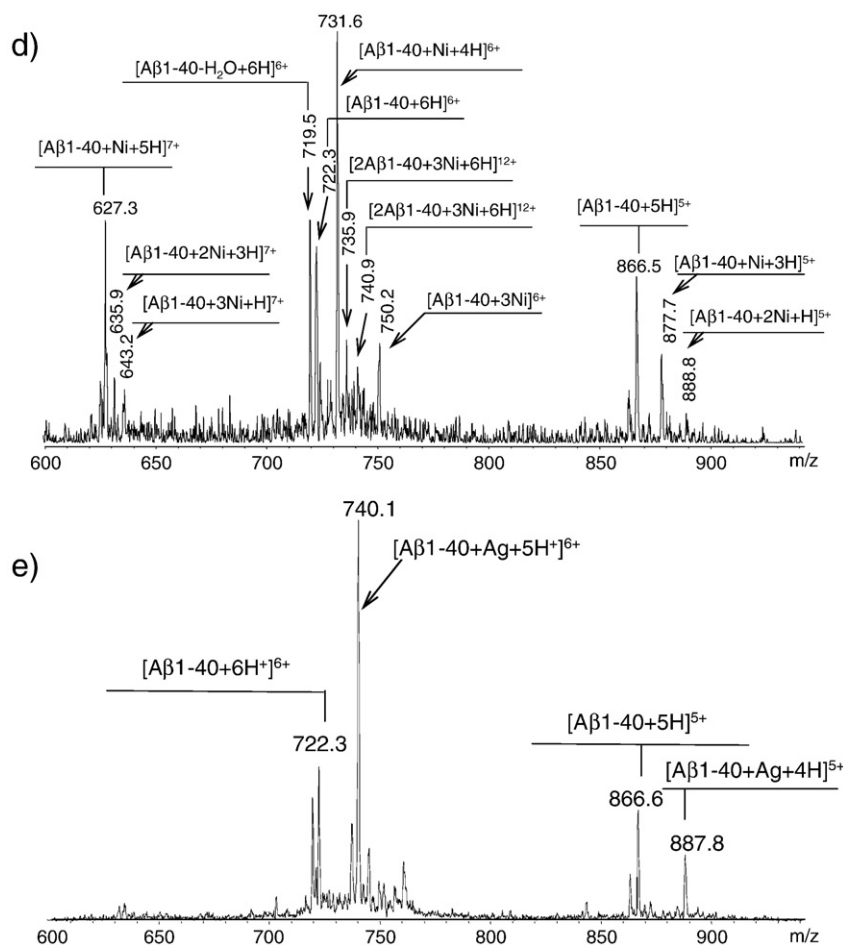
d)

731.6

$[A\beta1\text{-}40+Ni+4H]^{6+}$

$[A\beta1\text{-}40\text{-}H_2O+6H]^{6+}$

$[A\beta1\text{-}40+6H]^{6+}$

719.5

722.3

$[A\beta1\text{-}40+Ni+5H]^{7+}$

$[2A\beta1\text{-}40+3Ni+6H]^{12+}$

627.3

735.9

$[2A\beta1\text{-}40+3Ni+6H]^{12+}$

$[A\beta1\text{-}40+5H]^{5+}$

$[A\beta1\text{-}40+2Ni+3H]^{7+}$

740.9

866.5

$[A\beta1\text{-}40+Ni+3H]^{5+}$

635.9

$[A\beta1\text{-}40+3Ni+H]^{7+}$

750.2

$[A\beta1\text{-}40+3Ni]^{6+}$

877.7

$[A\beta1\text{-}40+2Ni+H]^{5+}$

643.2

888.8

600   650   700   750   800   850   900   m/z

740.1

e)

$[A\beta1\text{-}40+Ag+5H^+]^{6+}$

$[A\beta1\text{-}40+6H^+]^{6+}$

$[A\beta1\text{-}40+5H]^{5+}$

722.3

$[A\beta1\text{-}40+Ag+4H]^{5+}$

866.6

887.8

600   650   700   750   800   850   900   m/z

Fig. 1 (*continued*).

complex spectra were much simplified and some extra information was obtained.

3.6.1. Copper binding to amyloid-β peptide at pH 8.0

A slight increase in pH dramatically stimulated copper ion binding to Aβ1-40 (Fig. 4). Thus, Aβ1-40 peptide bound only one copper ion at

pH 6.6 (Fig. 4,a), whereas species with one, two and even three copper ions were determined at pH 8.0 (Fig. 4,b).

3.6.2. Zinc binding

Although the mass spectrum of Aβ peptide treated with 10:1 molar ratio zinc ions seems to be very simple, when enlarged each peak of
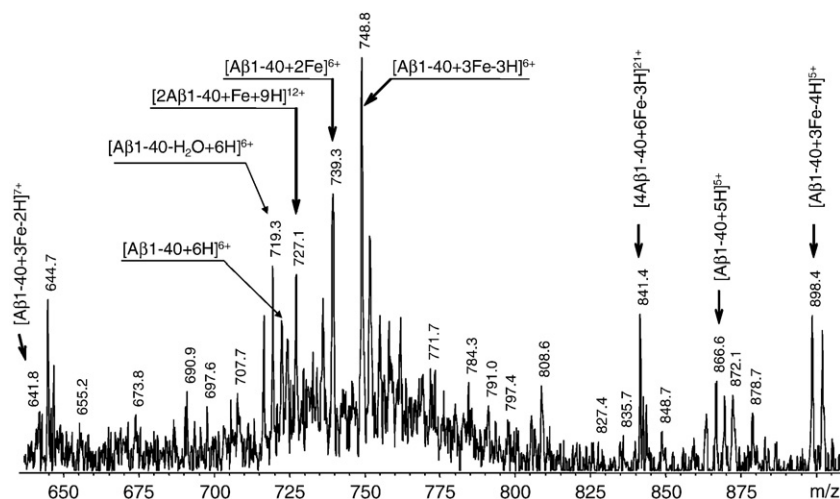
748.8

$[A\beta1\text{-}40+2Fe]^{6+}$

$[A\beta1\text{-}40+3Fe\text{-}3H]^{6+}$

$[2A\beta1\text{-}40+Fe+9H]^{12+}$

739.3

$[4A\beta1\text{-}40+6Fe\text{-}3H]^{21+}$

$[A\beta1\text{-}40\text{-}H_2O+6H]^{6+}$

841.4

$[A\beta1\text{-}40+5H]^{5+}$

719.3

727.1

$[A\beta1\text{-}40+3Fe\text{-}4H]^{5+}$

$[A\beta1\text{-}40\text{-}3Fe\text{-}2H]^{7+}$

644.7

$[A\beta1\text{-}40+6H]^{6+}$

898.4

641.8

771.7

784.3

866.6

655.2

673.8

690.9

697.6

707.7

791.0

797.4

808.6

827.4

835.7

848.7

872.1

878.7

650   675   700   725   750   775   800   825   850   875   m/z

Fig. 2. Species of iron(III) ion–Aβ1-40 complex at pH 5.5, as displayed in a truncated ESI ion trap mass spectrum. Free Aβ1-40 peptide ions (6+, m/z 722.3; 5+, m/z 866.6) may coexist with complexes containing three iron ions (m/z 641.8; 748.8; 898.4), two (m/z 739.3) or other various ones. Oligomerization is also possible (m/z 727.1).
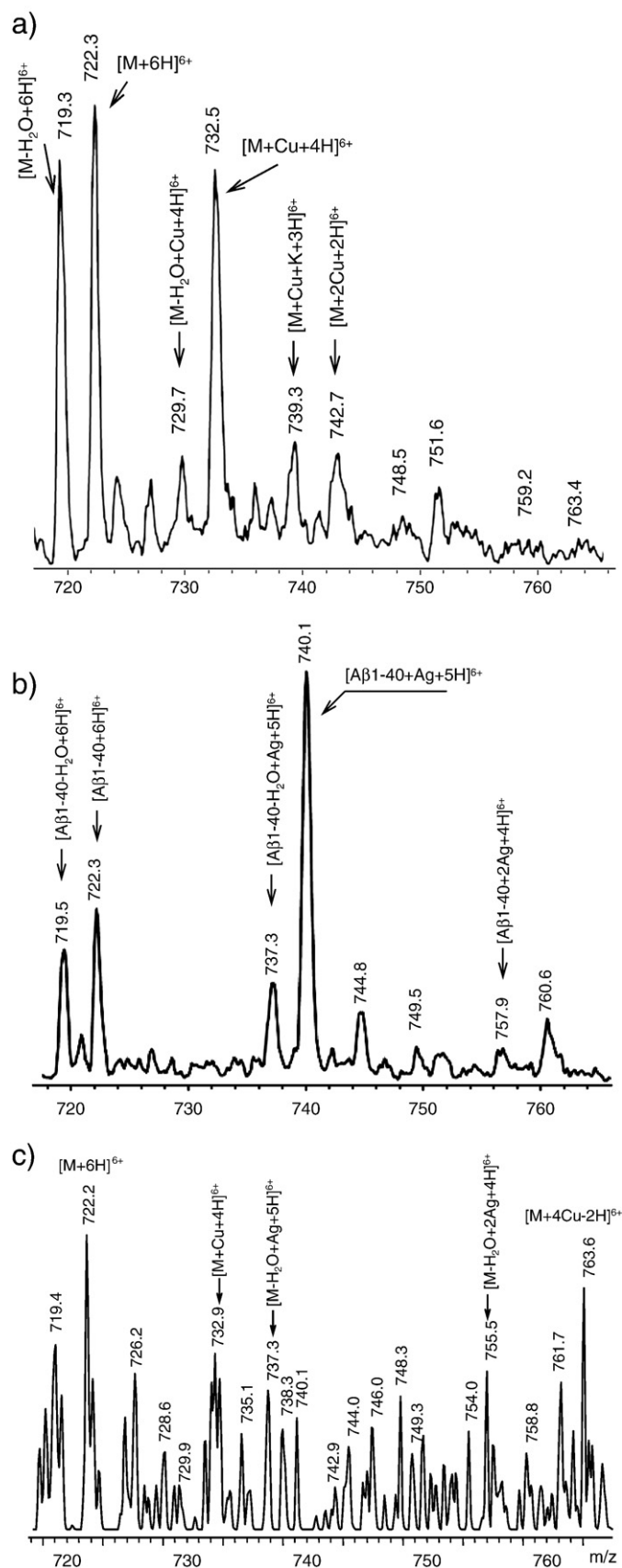
Fig. 3. Binding competition or synergism between copper ions and silver ones to Aβ1-40 sites at pH 5.5: a) copper binding to Aβ; b) silver binding to Aβ; c) copper and silver binding to Aβ.

this one becomes a complex group of signals belonging to various Zn (II)–Aβ species. The characteristic signals of Aβ1-40 practically disappeared and specific signals of Aβ molecules complexes with from two to ten zinc ions appeared in the mass spectrum (Fig. 5,a). For example, the major peak at m/z 638.0 was assigned to $[M + 2Zn + 5H]^{7+}$, being accompanied by the other signals of this molecular complex at m/z 203.9 (22+), 248.8 (18+), 344.2 (13+) and 447.0 (10+). Searching for metal-richer complexes of Aβ peptide, we found a well expressed complex of one molecule of Aβ with six ions of zinc; its major peak was displayed at m/z 337.1 (14+), and the others at m/z 214.8 (22+), 363.0 (13+), 428.9 (11+), 523.8 (9+), and 941.8 (7+), respectively. In addition, the signal at m/z 355.0 (14+) was assigned to $[M + 10Zn–6H]^{14+}$. Whereas the peak at m/z 638.0 was assigned to one Aβ1-40 molecule bound to two zinc ions, the signal at m/z 637.0 (8+) revealed the presence of Aβ peptide bound to 12 zinc ions. The existence of this complex was also confirmed by the most intense signal in the group around m/z 850.0 (m/z 848.9, $[M + 12Zn–18H]^{6+}$) as well as by the peaks at m/z 424.9 (12+), 636.9 (8+), or (727.8 (7+). Although the m/z 203.9 signal was associated with Aβ1-40 + 2Zn complex (22+), it also belongs to $[M–H_2O + 12Zn + H]^{25+}$ ion, and therefore this signal was found highly intensive, being the sum of two independent signals.

The most intense signal at m/z 211.9 was assigned to $[M + 5Zn + 12H]^{22+}$, so that, of so many peaks in the spectrum, the majority of them belongs to monomolecular Aβ complexes with more than one ion of zinc.

3.6.3. Nickel binding

The characteristic signals for Aβ peptide practically disappeared and various groups of new signals revealed a complex pattern of nickel binding to peptides (Fig. 5,b). In aqueous solutions of nickel salts and Aβ1-40 peptide found in a molar ratio of 10:1, either single molecules of Aβ bound one to more nickel ions or Aβ oligomers bound nickel in various proportions. First, we identified the simplest complex of Aβ1-40 with nickel, $[M + Ni + 4H]^{6+}$, at m/z 732.0 and 209.9 (21+) or 232.0 (19+). Nevertheless, one molecule of Aβ proved to bind more than five ions of nickel. For example, the peak at m/z 203.9 could be assigned to six nickel ions bound to Aβ1-40. However, nickel ions seem to be able to form oligomers when bound to peptides; the highest peak in the spectrum, m/z 355.1, was assigned to a metal–peptide complex containing three molecules of Aβ1-40 and two nickel ions. The existence of [3 M + 2Ni] complex was also confirmed by the signals at m/z 655.9 (20+), 546.9 (24+) and others. Even a single ion of nickel may induce oligomerization of Aβ peptide. An Aβ1-40 trimer bound to a nickel ion was identified at m/z 207.9, 221.9, 225.8, 622.0, etc. On increasing the pH and metal ion:peptide ratio, the spectra pattern became complicated dramatically. To avoid the assignment of some m/z values to false species, we referred only to the most evident complexes of Aβ with the investigated metal ions as revealed by the significant signals in the spectra. In addition, we increased stepwise both pH and metal ion concentration and followed the appearance of the new peaks that prove the formation of new species.

3.6.4. Silver binding

Generally, silver ions bound to individual molecules of Aβ1-40, without forming oligomeric species. Aβ1-40 peptide without one

Table 1
The main molecular ions identified in Fig. 3,c.

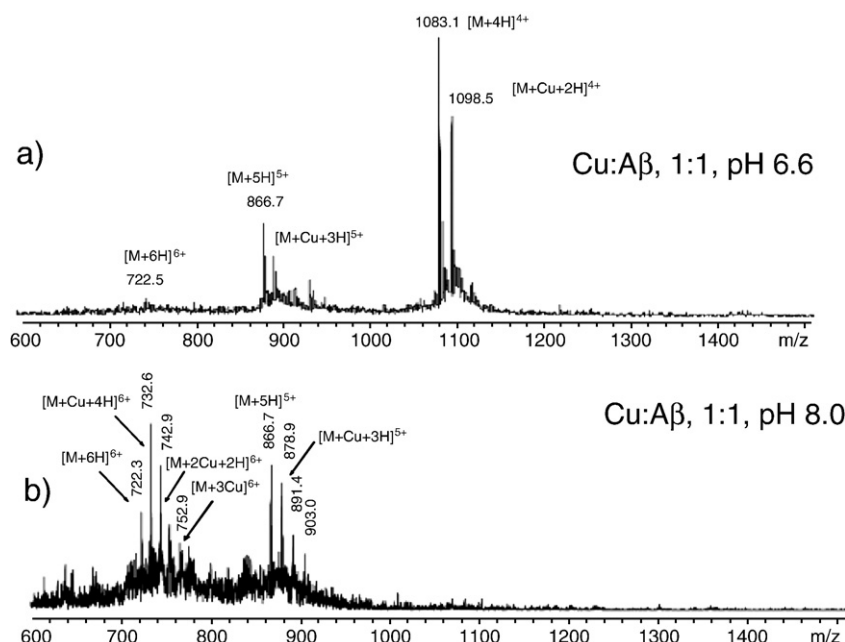| m/z	Molecular ion	m/z	Molecular Ion
719.4	$[M–H_2O + 6H]^{6+}$	740.1	$[M + Ag + 5H]^{6+}$
722.2	$[M + 6H]^{6+}$	742.9	$[M + 2Cu + 2H]^{6+}$
726.2	$[M + Na + 5H]^{6+}$	744.0	$[M + Ag + Na + 4H]^{6+}$
728.6	$[M + K + 5H]^{6+}$	749.3	$[M + Cu + Ag + 3H]^{6+}$
729.9	$[M–H_2O + Cu + 4H]^{6+}$	754.0	$[2 M + 3Cu]^{6+}$
732.9	$[M + Cu + 4H]^{6+}$	755.5	$[M–H_2O + 2Ag + 4H]^{6+}$
737.3	$[M–H_2O + Ag + 5H]^{6+}$	761.7	$[M–H_2O + 4Cu-2H]^{6+}$
738.3	$[2M + 3Cu]^{6+}$	763.6	$[M + 4Cu-2H]^{6+}$

Fig. 4. Effect of pH on copper binding to Aβ1-40: a) ESI ion trap mass spectrum of copper ion–Aβ1-40 peptide complex at pH 6.6, and b) MS spectrum of the complexes formed at pH 8.0. The molar ratio of Cu(II):Aβ was 1:1.

molecule of water proved to bind easily eight silver ions to form an Aβ complex that displayed specific signals in the mass spectrum at m/z 1034.5 (5+), as well as at m/z 862.4 (6+), 739.2 (7+), 575.2 (9+), 370.1 (14+), 324.1 (16+), 305.1 (17+), 288.1 (18+), 273.1 (19+), 247.0 (21+), and 236.0 (22+), respectively. Another peak at m/z 1035.4 was assigned to $[M+8Ag-3H]^{5+}$, which was less intensive than that of $[M-H_2O+8Ag-3H]^{5+}$. This complex of Aβ1-40 with eight silver ions was also supported by characteristic signals at m/z 863.2 (6+), 518.2 (10+), 471.1 (11+), 432.2 (12+), 226.0 (23+), and 208.0 (25+), respectively.

The peak at m/z 216.0 (23+) also appeared at m/z 990.4 associated with $[M-H_2O+6Ag-H]^{5+}$ complex, and consequently, it was assigned to Aβ1-40 complex with six silver ions, being present at m/z 413.1 (12+), and m/z 226.0 (22+).

Looking for metal-richer complexes of Aβ1-40, we found a signal at m/z 689.3, which could be assigned to $[M+11Ag-3H]^{8+}$. However, first we calculated the molecular weight of this complex and looked for its five charged ion (m/z 1102.5). Following this procedure, we identified the other peaks of Aβ1-40 molecules, each of them containing eleven silver ions, such as m/z 788.3 (7+), 368.0 (15+), 345.1 (16+), 325.1 (17+), 307.1 (18+), and 263.1 (21+), respectively. Aβ1-40 proved to bind also 12 Ag ions to each molecule as revealed by the relatively less intense signals at m/z 1124.4 (5+), 937.3 (6+), 703.3 (8+), 625.2 (9+), 469.2 (12+), 433.2 (13+), 402.1 (14+), 352.1 (16+), 313.1 (18+), 235.0 (24+), 217.0 (26+), and 209.0 (27+).

Finally, the highest peak in the spectrum at m/z 651.2 was assigned to $[M+2Ag+5H]^{7+}$. Hence, treating Aβ1-40 molecules with silver ions in the 1:10 molar ratio, numerous species of silver–peptide complex were found, whereas Aβ adduct with eight silver ions was prevalent. To explain the formation of such a large number of silver–Aβ complexes, we took into consideration the participation of both histidine residues and free amino group as well as carboxylate ones in the binding process.

3.7. Nickel–silver competition for Aβ1-40 binding sites at basic pH

Treating Aβ1-40 in the presence of triethanolamine with a solution containing both silver and nickel ions, a competition among these ions was observed (Fig. 6). Apparently, the spectrum of the silver–nickel–

Aβ1-40 peptide system became simplified as compared to those of both silver–Aβ and nickel–Aβ complexes. Indeed, the main signals in this spectrum were only found at 355.0 (100%), 203.9 (77.8%), 205.9 (72.2%), 411.0 (40.4%), 357.0 (39.1%), 413.0 (33.0%), 337.1 (21.7%), 618.0 (18.9%), and 653.9 (10.9%), respectively. However, contrary to the current spectra of peptides or metal ion–peptide complexes at neutral pH, each group of signals in a given area of the spectrum of metal ion–peptide complexes could not be assigned to an isotopic distribution but to many individual complexes. For example, the major signal at m/z 355.0 consists of a group of two intense peaks and three lower ones, ranging from m/z 355.0 to m/z 359.0. The peak at m/z 355.0 was assigned to $[M+6Ag+8H]^{14+}$ ion, that at m/z 356.0 to $[M+11Ag+3Ni-H]^{16+}$, whereas the signal at m/z 357.0 (calcd. m/z 357.2) was assigned to $[M+8Ag+3Ni+H]^{15+}$, and m/z 358 could be assigned to $[M+3Ag+10H]^{13+}$, although they are differently charged and contain various ratios between the metal ions.

Therefore, we searched first for the signals' characteristic to 1:1 molar ratio Ni–Aβ complexes. Indeed, one can find low signals, at the detection limit, for one nickel ion bound to a single molecule of Aβ1-40 at m/z 731.9 (6+), 488.3 (9+), 338.3 (13+), 231.9 (19+), and 209.9 (21+) only. Nevertheless, trace signals at m/z 741.2 (6+), 635.5 (7+), 371.0 (12+), and 247.7 (18+) could be attributed to Aβ1-40 complex with two nickel ions. In addition, the existence of 3Ni:Aβ1-40 molar ratio complex was demonstrated by the signals at m/z 750.8 (6+), 563.3 (8+), 451.0 (10+), 410.0 (11+), and 250.9 (18+). We also found 4Ni:Aβ140 complex at m/z 651.9 (7+), 506.9 (9+), 414.9 (11+), and 207.9 (22+) as well as species containing five nickel ions in a molecule at 771.1 (6+), 660.9 (7+), 385.9 (12+), and 201.9 (23+), respectively.

No other signals for nickel ions bound to Aβ were found except those characteristic to 6Ni:Aβ1-40 molar ratio: 780.9 (6+), 586.0 (8+), 361.0 (13+), and 223.9 (21+). We also searched for signals according to their calculation using masses for the expected complexes. Thus, we found characteristic signals for Ag:Aβ, 2Ag:Aβ, or for Ag:Ni:Aβ, 2Ag:Ni:Aβ, etc.

Nevertheless, each signal in a small range of m/z values consists of many signals, which can be assigned to different silver–Aβ complexes, or silver–nickel–Aβ ones. We found a complex with MW 4919.4 composed of one molecule of Aβ1-40, five silver ions and
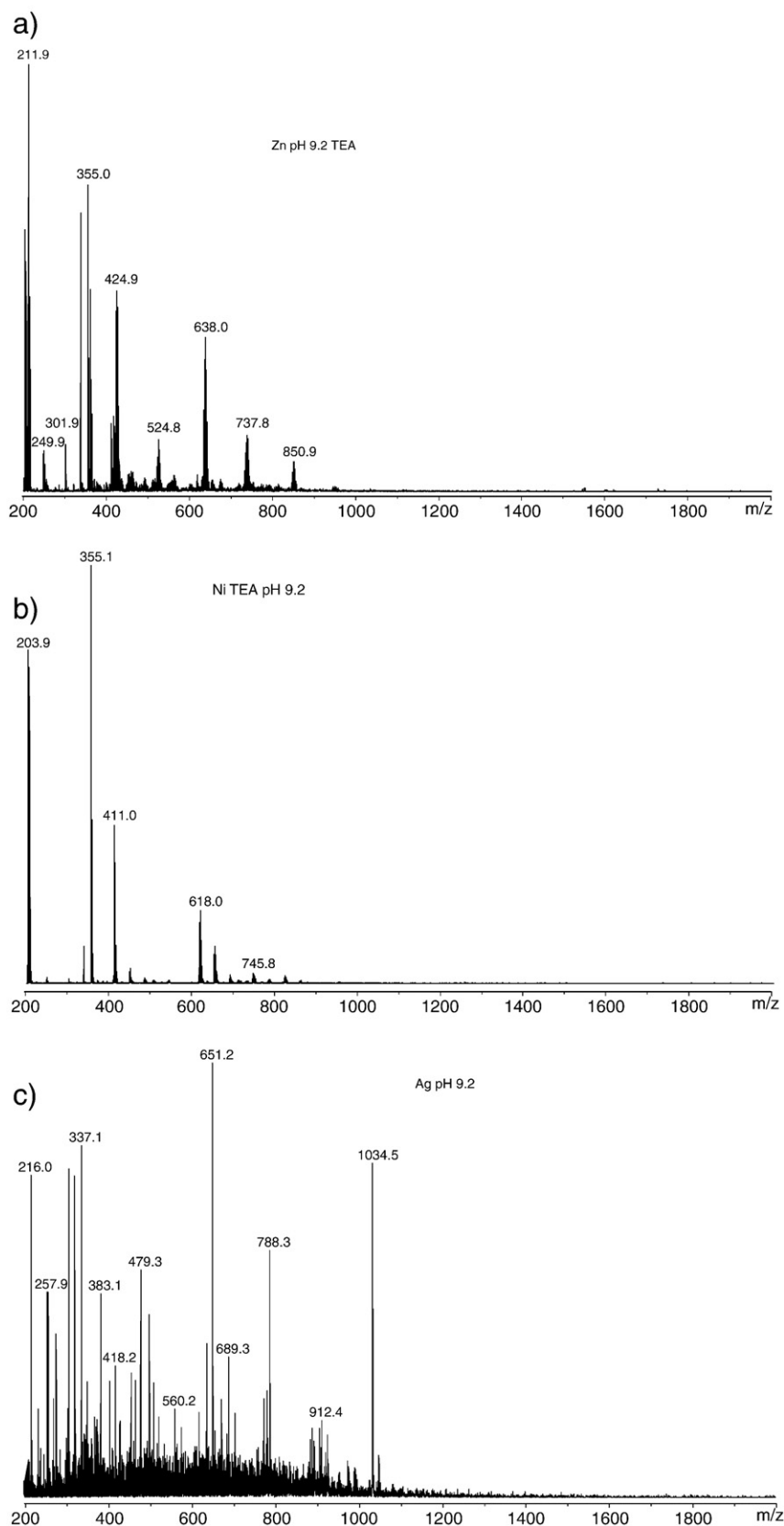
Fig. 5. Metal ion binding to Aβ peptide at pH 9.2: a) ESI ion trap mass spectrum of Ni(II)–Aβ1-40 peptide complexes; b) Zn(II) binding to Aβ at pH 9.2; Ag(I)-Aβ1-40 complexes as revealed by MS.

one nickel ion. Its characteristic signals were also found at m/z 820.9 (6+), 616.0 (8+), 448.5 (11+), 411.0 (12+), and 205.9 (24+), respectively.

The main signal in the area from m/z 201.9 to 209.9 was that at m/z 203.9, which was assigned to $[M + 11Ag + 3Ni + 11H]^{28+}$, corresponding to a silver–nickel-Aβ1-40 complex with the molecular weight of
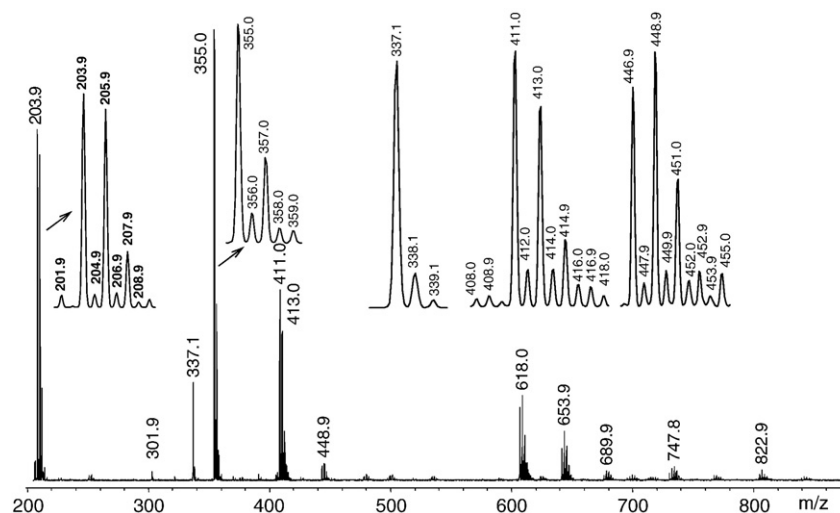
Fig. 6. Competition and synergism between silver and nickel ions within the process of metal ion binding to Aβ1-40 peptide at pH 9.2.

5678.0 (calcd. 5678.6). Its existence was also supported by the other peaks at m/z 710.9 (8+), 632.0 (9+), 356.0 (16+), and 247.9 (23+), respectively. We clearly stated that generally high molecular mass complexes give signals at lower m/z values due to their highly charged ions, whereas the low molecular peptides and metal ion–peptide complexes at higher ones.

3.8. Circular dichroism investigation

The influence of metal ions on the secondary structure of the Aβ1-40 peptide was followed by CD spectroscopy. Metal ion complexation has previously been shown to be a strong trigger for a secondary structure switch from an a-helix to a β-sheet [24]. The CD spectra of Aβ1-40 peptide (180 mM) in aqueous solutions or in the presence of copper ions, sodium dodecylsulfate and nickel ions at two pH values are shown in Fig. 7. The CD spectrum of the peptide showed a low degree of ordering (49.1% unordered forms and 34.3% β-sheet conformers), which was not drastically modified by the addition of copper ions. However, a slight increase in the pH value enhanced the
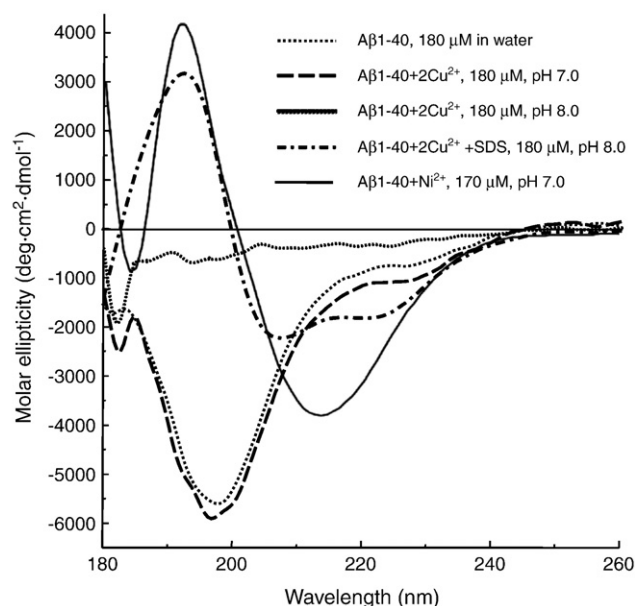
proportion of β-sheet conformers (up to 40.2%). In the presence of SDS, the conformational equilibrium changed from unordered and β-turn structures to β-sheet (46%) and a-helix conformers (Table 2, Fig. 7). Some other measurements showed that SDS produces similar conformational equilibriums of Aβ1-40 species that are independent on the pH values and copper ion concentrations. SDS induced a complete abolition of β-turn conformers and an increase in the population of β-sheet conformers (Table 2). To explain the atomization of Aβ aggregates by SDS, even if it produces an increasing in β-sheet proportion of Aβ1-40 molecules, we considered the hydrophobic interactions between SDS molecules and the hydrophobic amino acid residues of Aβ1-40 molecule, which may induce a shift toward α-helical conformers which behave different from fibrillogen β-sheet ones.

Nickel ions changed dramatically the secondary structure of Aβ1-40 from a large proportion of random coil (49.1%) to one of α-helix conformers (25.3%). In addition, nickel induced a slow decrease in the population of β-turn conformers, and a slight increase in that of β-sheet species (38.5%). These findings are concordant with the MS measurements, which showed the formation of various nickel–Aβ1-40 complexes containing variable molar ratios between Aβ and nickel.

By addition of equimolecular amounts of iron ions, the conformational equilibrium was almost unchanged (43.7% unordered conformers and 37.5% β-sheet ones). Iron induced a very slow increase in the population of beta conformers, that are related to Aβ toxicity and Alzheimer's pathogenesis. In contrast to copper ions, which increase the proportion of β-sheet conformers, or nickel ions, which induce the formation of β-turn species, Fe(III) had little effect on the conformational equilibrium. However, hydrophobic environments, such as that generated by SDS, increased α-helical proportion, which is supposed to reduce β-sheet toxicity.

Fig. 7. CD spectra of Aβ1-40 peptide and of its complexes with copper and nickel ions at pH 7 and 8 and in the presence of sodium dodecyl sulfate (SDS; 1:1 molar ratio).

Table 2
Conformational changes of β-amyloid peptide (Aβ1-40 or Aβ) in the presence of copper ions (Cu), nickel ions (Ni), and sodium dodecyl sulfate (SDS) at pH 7.0 and pH 8.0.

Conformation (%)	Aβ1-40	Aß/Cu/pH 7	Aß/Cu/pH 8	Aß/Cu/SDS/pH 8	Aß/Ni/pH 7
α-helix	0.0	0.0	0.0	22.8	25.3
ß-sheet	34.3	34.7	40.2	46.0	38.5
ß-turn	16.6	17.0	16.6	0.0	9.1
Random coil	49.1	48.2	43.2	31.2	27.2
Total	100.0	100.0	100.0	100.0	100.0
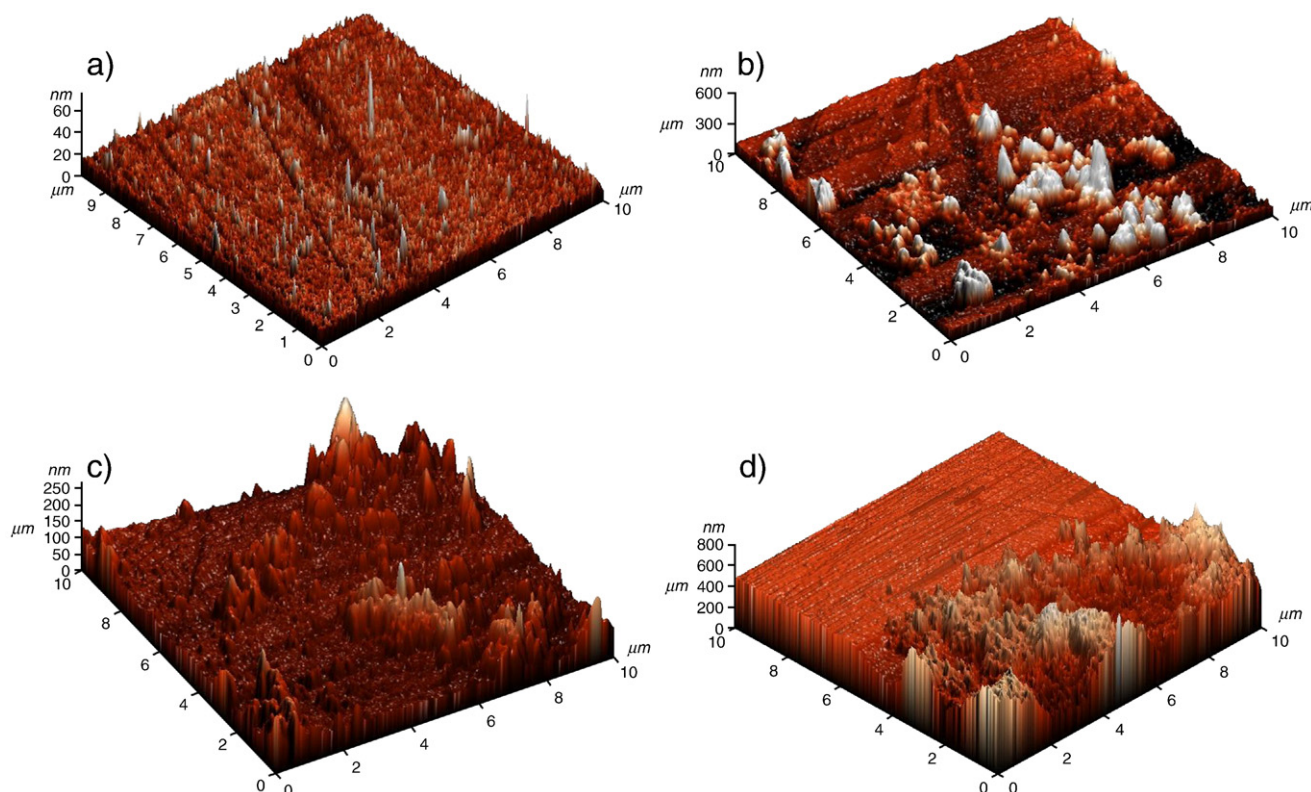
Fig. 8. AFM images (10 μm × 10 μm) of Aβ1-40 and its 1:10 molar ratio adducts: a) Aβ1-40; b) Aβ1-40 + Cu2 +; c) Aβ1-40 + SDS; d) Aβ1-40 + SDS + Ag+.

3.9. Atomic force microscopy investigation

The self-assembled nanostructures of peptides in aqueous solution were significantly influenced by the presence of metal ions (Fig. 8). Fresh solution of Aβ1-40 formed a smooth film on the glass plate of the microscope. Copper ion binding to Aβ1-40 peptide plays an important role in the formation of nanostructures as shown in Fig. 8,b. Copper ions thus generated a rough surface containing numerous big structures as high as 200 nm. It is also clear from this image that copper ions are not able to induce large nanostructures, but small hills. However, the strong contrast between the AFM images recorded before and after copper treatment confirmed a morphological change in the surface packing of the peptide shell. Although SDS do not form non-covalent complexes with Aβ1-40, the high roughness of the surface in Fig. 8,c showed an evident interaction of the two molecules, which results in the formation of supramolecular structures. On treating copper–Aβ1-40 complex with SDS, similar nanostructures are created from these adducts. Similarly, silver–Aβ1-40 film differed dramatically from that of Aβ1-40 one, as well as that of the solution containing silver ions, Aβ1-40 molecules and SDS (10:1:10 molar ratio; Fig. 8,d).

4. Discussion

The stoichiometry and binding mode of Aβ1-40 peptide showed a great variety depending on the metal ion to ligand ratio, and pH. These results are concordant with those obtained previously on working with individual metal ions [29–32]. Although blood and internal biochemical environment is characterized by a pH close to 7.39 (7.35–7.45), most fluids inside the body as well as body tissues function at different pH ranges [33,34]. Under various physiological conditions, acutely dissociated neurons were also found to have a wide range of pHi values (6.3–7.7) [35,36]. During near-complete rat forebrain ischemia, cerebral intracellular pH decreased from 7.01 to 6.17 [37]. This was the incentive to investigate the interaction of metals with peptides within a large pH

range. The coordination of metal ions to Aβ is the same in both aqueous solution and lipid environments, with His[6], His[13], and His[14] all involved [19]. However, the peptide backbone becomes more important in metal ion-binding process on increasing pH. Nickel ions bind strongly to Aβ1-40 even in acidic solutions. Nevertheless, silver ions enhance copper binding to Aβ in acidic solutions, whereas they compete with nickel ions at higher pH.

The generation of the Aβ toxic species is modulated by the Cu^{2+} concentration and the ability to form an intermolecular His bridge as demonstrated by EPR spectroscopy [29]. Several spectroscopic studies have indicated that the copper-binding site of Aβ consists of the three histidine residues His6, His13 and His14 and an as yet undefined fourth ligand; proposed ligands include Tyr10 [19,38–40], the N-terminal nitrogen [30] or an as yet unidentified carboxylate side chain [31,32].

Our study revealed a high affinity of Aβ toward iron(III) ions, with which Aβ forms both iron-bound oligomers and high molecular weight polymeric species. They might have a high patho-physiological significance because pH and reduction-oxidation balance of certain cells may alter alongside many pathological processes.

Depending on the pH values, zinc ions may bind to Aβ peptide in various proportions as our investigation revealed. Although protein chelation of zinc is typically mediated by histidines, cysteines and carboxylates at low pH [41], amino groups may play an important role under slight alkaline conditions.

Further investigation should be done to clarify the effect of metal ions on the conformational changes of Aβ as well as their interference with the other metal ions within the binding process. Aβ(1–16) was previously shown to bind up to three cations upon increasing the Zn^{2+} concentration [2]. Under CID, zinc binding induces specific cleavages after the three histidines of the Aβ(1–16) sequence (H[6], H[13] and H[14]), showing their simultaneous implication in the Zn^{2+} coordination sphere [2]. In addition, Glu11 residue has been identified as providing the carboxylate side chain when Zn^{2+} is bound to Aβ1-16 [42].

Circular dichroism spectroscopy and electron microscopy were used as well to monitor the effects of metal ions on the peptide β-sheet

conformation and fibril aggregation. These observations indicated a specific zinc binding site within Aβ that involves these central histidine residues. Complementary studies with Cd^{2+} ions cause perturbations to ^1H NMR spectra of Aβ(1–40) that are strikingly similar to that observed for Zn^{2+} [18]. The binding of divalent metals like Zn^{II}, Cd^{II} and Co^{II} to a synthetic peptide, $APP^{170-188}$ induces the dimerisation of the peptide [43].

Metal ions have been suggested to induce aggregation of amyloid beta-peptide, which is a key event in Alzheimer's disease [44]. Although, histidine-containing peptide fragments of prion protein are efficient ligands to bind various transition metal ions with the following order of metal affinity [45]: $Pd(II) \gg Cu(II) \gg Ni(II) \geq Zn(II) > Cd(II) \sim Co(II) > Mn(II)$, our study showed the nickel was bound by Aβ with higher affinity than copper ions. The high selectivity of metal binding is connected to the involvement of both imidazole and amide nitrogen atoms in metal binding for various metal ions [45].

Our results showed that Aβ peptide is helical in hydrophobic environments and becomes mostly β-sheet in aqueous media or in the presence of some metal ions, which is concordant with Lau et al. [46]. Glycine residues in Aβ1-40 may be important in metal binding because they allow an appropriate folding of the peptide. Indeed, the GxxxG motifs of Aβ is a key structural and functional determinant [47]. Besides, β-amyloid protein oligomers induced by metal ions and acid pH are distinct from those generated by slow spontaneous ageing at neutral pH, as previously shown [48].

The three techniques used here (ESI-MS, CD, and AFM) revealed the complex interaction between Aβ1-40 peptide with various metal ions under different pH conditions, with possible impact on the research methodology in various pathologies.

5. Conclusions

We have reported here a complex pattern of metal–metal competition for Aβ1-40 binding sites, which depend essentially on the involved metal ions, their concentration, and pH changes. Thus, metal ions bind specifically to Aβ1-40 peptide and change dramatically its conformation. Increasing pH results in increasing metal ions by Aβ. Significant copper and zinc bind to Aβ1-40 peptide at pH 5.5. However, up to three nickel ions commonly bind to each molecule of β-amyloid peptide, whereas the complex of Aβ1-40 with four nickel ions was also confirmed by low signals at m/z 760.6 (6+) and 652.1 (7+). In addition, nickel ions proved to enhance Aβ oligomerization. Nevertheless, with increasing pH, up to 12 ions of zinc may bind to a single Aβ molecule. Both silver and iron ions displayed different binding patters as compared to the other investigated metal ions. Aβ1-40 peptide without one molecule of water proved to bind easily eight silver ions to form an Aβ complex that displayed specific signals in the MS spectrum at m/z 1034.5. Iron(III) ions produced both the oligomerization of the peptide and the formation of iron-rich peptide complexes. Under the same pH and concentration conditions, the binding pattern of the independent copper and silver ions to Aβ1-40 was different from that of the equimolecular mixture of the two metal ions. Maybe, some conformational changes due to water loss altered the capacity of Aβ peptide to bind certain heavy metal ions. As a consequence, copper-silver interaction with the binding process to Aβ1-40 was highly complex. A competition between silver and nickel ions at high pH for Aβ1-40 binding sites was also observed. New strategies were proposed to identify the characteristic signals for some important metal ion-peptide complexes in the spectra recorded at high pH or high metal concentrations. Unlike the current spectra of peptides or metal ion-peptide complexes at neutral pH, each group of signals in a given area of the spectrum of high metal ion-peptide complexes at higher pH cannot be assigned to an isotopic distribution but many individual complexes. To explain the formation of such a large number of high metal-Aβ complexes, we took into consideration the participation of both

histidine residues and free amino group as well as carboxylate ones in the binding process. Circular dichroism and atomic force microscopy studies supported the mass spectrometric data.

Further research is needed to clarify the significance of multiple interactions of metal ions with Aβ1-40 at various pH values for AD pathology.

Acknowledgements

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