

# Effect of metal chelators on the aggregation of beta-amyloid peptides in the presence of copper and iron

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**Abstract** Amyloid  $\beta$  ( $A\beta$ ) fibrils and amorphous aggregates are found in the brain of patients with Alzheimer's disease (AD), and are implicated in the etiology of AD. The metal imbalance is also among leading causes of AD, owing to the fact that  $A\beta$  aggregation takes place in the synaptic cleft where  $A\beta$ , Cu(II) and Fe(III) are found in abnormally high concentrations.  $A\beta_{40}$  and  $A\beta_{42}$  are the main components of plaques found in afflicted brains. Coordination of Cu(II) and Fe(III) ions to  $A\beta$  peptides have been linked to  $A\beta$  aggregation and production of reactive oxygen species, two key events in the development of AD pathology. Metal chelation was proposed as a therapy for AD on the basis that it might prevent  $A\beta$  aggregation. In this work, we first examined the formation of  $A\beta_{40}$  and  $A\beta_{42}$  aggregates in the presence of metal ions, i.e. Fe(III) and Cu(II), which were detected by fluorescence spectroscopy and atomic force microscopy. Second, we studied the ability of the two chelators, ethylenediaminetetraacetic acid and 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol), to investigate their effect on the availability of these metal ions to interact with  $A\beta$  and thereby their effect on  $A\beta$  accumulation. Our findings show that Fe(III), but not Cu(II), promote aggregation

of both  $A\beta_{40}$  and  $A\beta_{42}$ . We also found that only clioquinol decreased significantly iron ion-induced aggregation of  $A\beta_{42}$ . The presence of ions and/or chelators also affected the morphology of  $A\beta$  aggregates.

**Keywords**  $\beta$  amyloid peptide · Cu(II) ion · Fe(III) ion · Clioquinol · EDTA

## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease described firstly by Alois Alzheimer in 1906 (Selkoe and Hardy 2016). AD is characterized by progressive dementia and abnormal extracellular amyloid-beta ( $A\beta$ ) peptide depositions in the form of senile plaques in the brain (Santos et al. 2016). In the elderly, AD is the most common cause of dementia (Sastre et al. 2015).  $A\beta$  appears in two forms,  $A\beta_{40}$  and  $A\beta_{42}$ , and are the main components of the plaques that are found in afflicted brains (Gu et al. 2016). Although the concentration of  $A\beta_{40}$  soluble monomer is more than  $A\beta_{42}$ ,  $A\beta_{42}$  is the main component of amyloid plaques (Gu et al. 2016).  $A\beta_{40}$  and  $A\beta_{42}$ , differ by two residues at the C-terminus (Cukalevski et al. 2015). These two hydrophobic residues (Ile and Ala), make  $A\beta_{42}$  more aggregation prone than  $A\beta_{40}$  (Cukalevski et al. 2015). The amyloid beta peptides are cleaved from amyloid

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precursor protein (APP) by two secretase enzymes, called  $\beta$ - and  $\gamma$ -secretases (Wallin et al. 2016). Oligomers, amyloid fibrils, protofibrils and diffuse plaques (non-fibrous aggregates) of amyloid beta peptides such as amorphous aggregates are also found in the brain (Jiang et al. 2012). A $\beta$  oligomers are more cytotoxic than fibrillary aggregates (Jamasbi et al. 2016). Oligomers have a wide molecular weight range (from <10 to >100 kDa) (Sakono and Zako 2010).

Decreasing synapse number, inhibiting long-term potentiation in the hippocampus of rodent and memory loss in rats are the results of A $\beta$  oligomer injections into their healthy brains (Selkoe and Hardy 2016).

Copper and iron ions play pivotal roles in the biological processes in some regions of the brain (Wineman-Fisher et al. 2016). According to previous studies, high concentrations of Cu(II) (400  $\mu$ M) and Fe(III) (1  $\mu$ M) are co-localized within the amyloid plaques in the brain and may be involved in their formation (Orvig et al. 2012).

These redox-active metal ions can bind to A $\beta$  and may produce free-radicals or reactive oxygen species (ROS) that are the major source of oxidative stress (Orvig et al. 2012). According to a recent study, they bind to the His13 and His14 residues of A $\beta$  peptides in such a way that the relative strength of the binding of iron to above residues is more than that of copper and zinc (Kong et al. 2015).

Metal ion chelation has been proposed as a novel therapeutic strategy for Alzheimer's disease (Santos et al. 2016). The field of metal chelation in AD continues to expand rapidly (Santos et al. 2016). Treating AD by chelation therapy should primarily involve a low molecular weight, lipophilic metal chelator to cross the blood–brain barrier (BBB), bind metal ions, and thereby prevent A $\beta$  aggregation (Prachayasittikul et al. 2013). Clioquinol (CQ) (one of the metal chelators that was used in this study) is a small lipophilic anti-amyloid drug that binds non-specifically to Cu(II) or Zn(II) in a 2:1 ligand: metal ratio. The affinity constant of CQ for Cu(II) is only one order of magnitude higher than that for Zn(II) (log  $K = 10$  and  $9$ , respectively) (Robert et al. 2015). CQ can bind to iron to a lesser extent than copper (Matlack et al. 2014). It is a bidentate chelating agent that forms 2/1 (ligand/metal ratio) square planar complexes and is able to cross the BBB (Lim et al. 2009). Ethylenediaminetetraacetic acid (EDTA), a hexadentate

chelator that does not cross the BBB and forms especially strong complexes with Cu(II) and Fe(III) (also with ions such as Mn(II), Pb(II) and Co(III)), was used to see the effect of using another chelator (without considering its potential therapeutic use) on aggregate formation. In this way we would presumably better ascribe any effect of CQ on A $\beta$  aggregation to its metal ion chelation rather than to its non-chelation chemical interaction with the peptide. Many reports about the effects of metals and chelators on amyloid aggregation have been published (Santos et al. 2016). It is proposed that metal chelators or better to say, metal protein attenuating compounds (MPACs), either might dissolve the deposits in the brain tissue or might prevent amyloid beta aggregation (Santos et al. 2016). It is reported that CQ can inhibit A $\beta$  accumulations and improve memory capacity in Alzheimer's disease transgenic mice (Robert et al. 2015).

Amyloid fibrils can be detected by fluorescence spectroscopy, with the use of thioflavin T (ThT), a specific dye with specific binding capacity to  $\beta$ -sheets (Faller et al. 2013). We used TFE to prepare relatively hydrophobic A $\beta$ 40 and A $\beta$ 42 stock solutions (modeling the hydrophobic environment of neuronal membrane) as TFE does not interfere with ThT fluorescence and it does not affect the ThT emission of A $\beta$  fibrils (Tiiman et al. 2015). Chloride salts of copper and iron ions were used because of their relatively easy solubilization in aqueous solutions and also because Cl<sup>-</sup> anion is abundantly present in biological fluids.

Dysregulation of copper and iron homeostasis is known to occur upon normal aging and in neurodegenerative diseases, specifically in AD (Robert et al. 2015). Consequently, it has been proposed that oxidative stress and release of reactive oxygen species could be the result of their dysregulation. But it is remained to be clarified if copper and iron dysregulation also promote A $\beta$  peptide aggregation? Here, we have tried to investigate this question.

In this work we have examined the effects of copper and iron ions on the aggregation process of A $\beta$ . Herein, we made use of fluorescence spectroscopy to provide evidence whether Cu(II) or Fe(III) can strengthen the ability of A $\beta$ 40 and A $\beta$ 42 to form ThT-positive structures or not. In addition, with the use of atomic force microscopy (AFM), we tried to visualize the aggregates (oligomers, fibrils, and

amorphous aggregates) that were formed in the presence of beta-amyloid peptides, alone, or in the presence of Fe(III) or Cu (II) ions. We also examined the presence of metal chelators (CQ and EDTA) in the reaction mixtures containing beta amyloid peptides and metal ions to investigate their effect on beta-amyloid aggregate formation. Selection of CQ was mainly because it can pass through BBB (contrary to EDTA) and consequently because of its therapeutic potential in AD.

## Materials and methods

### Chemicals

A $\beta$ 40 and A $\beta$ 42 peptides were purchased from Sigma and their stock solutions were prepared in TFE. All samples were prepared by diluting proper volumes of peptide stocks in 0.05 M Tris buffer solution, pH 7.4. Chloride salt solutions of Fe(III) and Cu(II), were purchased from Aldrich. ThT was from Sigma and its stock solution was prepared in distilled water. CQ was purchased from Fluka and ethanol was used to prepare its stock solution. The EDTA stock solution was prepared in distilled water.

### ThT fluorescence assay

In all experiments, the concentrations of A $\beta$ 40 and A $\beta$ 42 were 1  $\mu$ M. The final concentrations of Cu(II) and Fe(III) in the sample mixtures were held at 1  $\mu$ M (equimolar metal: peptide ratio), and were added before the start of incubation (25 °C for 24 h). The concentrations of EDTA and CQ in sample mixtures were 20  $\mu$ M and were also added at the beginning of the incubation. The  $\beta$ -sheet-rich content of amyloid aggregates was detected by ThT fluorescence spectroscopy. Experiments were triplicated and the data points were obtained by averaging. The fluorescence intensity of each sample was measured by excitation and emission at 440 and 480 nm (slit width 5 nm), respectively, with the use of a Cary Eclips VARIAN fluorescence spectrophotometer. To eliminate the interference effects of CQ and EDTA in chelator-containing samples during fluorescence measurements, we dialyzed the incubated samples for 12 h (10 KDa cut-off) and exchanged the buffer four times.

### Atomic force microscopy (AFM)

In order to confirm and visualize the presence of amyloid fibrils and study the morphology of aggregates, AFM technique was employed. 5  $\mu$ L of each sample was deposited onto a freshly cleaved mica slide, with a dimension of 1  $\times$  1 cm, and incubated for 30 min at room temperature. Samples were rinsed with 50–100  $\mu$ L deionized water to remove unbound protein and were left to dry at room temperature. The AFM images were obtained using a Veeco AFM instrument with the use of a non-contact mode AFM (nc-AFM). WSXM software was used to measure the size and diameter of aggregates from the Z-heights in AFM images (Horcas et al. 2007).

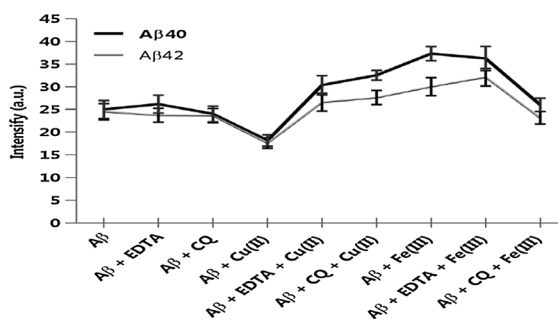
### Statistical analysis

Statistical analyses were carried out with GraphPad Prism version 7.00 software. Two-tailed Student's *t* test and two-way ANOVA test were used for differences between two groups. P-value < 0.05 was considered significant statistically. The data are presented as mean  $\pm$  SD of at least three independent experiments.

## Results and discussion

### Aggregation of A $\beta$ in the presence of metal ions

A $\beta$ 40 and A $\beta$ 42 in the absence of Cu(II) and Fe(III) ions formed ThT-reactive amyloid species. The fluorescence studies of samples containing only A $\beta$ 40 or A $\beta$ 42 resulted in the same intensities (25 a.u., Fig. 1).



**Fig. 1** ThT fluorescence of different mixtures of A $\beta$ 40 and A $\beta$ 42 at 480 nm (a.u)

In the case of A $\beta$ 40, the presence of Cu(II) (A $\beta$ 40 + Cu) decreased the intensity (17 a.u., Fig. 1) and that of Fe(III) (A $\beta$ 40 + Fe) increased considerably the intensity (37 a.u., Fig. 1). Effect of Cu(II) on A $\beta$ 42 (A $\beta$ 42 + Cu) was the same as that of A $\beta$ 40, i.e. 17 a.u., and the presence of Fe(III) (A $\beta$ 42 + Fe) also showed an increase, although the increase was mild in comparing with A $\beta$ 40 (29 a.u.). So, it seems that copper ions could not increase ThT-reactive species and iron ions showed increases, for both A $\beta$ 40 and A $\beta$ 42 peptides.

AFM images of beta amyloid peptide aggregates in the absence metal ions showed the presence of numerous oligomers with a diameter about 40–60 nm (Fig. 2I), although they were more numerous in A $\beta$ 42 samples (Fig. 2IB). Since their fluorescence intensities were the same (above paragraph) and since in ThT fluorescence, the beta structures are detected, it seems that amorphous aggregates were more numerous in A $\beta$ 42 samples. Some small clusters of individual amyloid fibers and amorphous aggregates were evident in A $\beta$ 40 sample (Fig. 2IA). As it was stated above, the presence of iron increased the fluorescence intensities in both A $\beta$ 40 and A $\beta$ 42 samples. AFM results confirmed this observation by the appearance of a considerable number of fibrillar structures and disperse aggregates with denser fibrillar and prefibrillar aggregates in the samples containing Fe(III). Figure 2III shows the effect Cu(II) on the extent and morphology of aggregates. A $\beta$ 40, in the presence of Cu(II) ions, showed more affinity to form amorphous aggregates and oligomers with a diameter of about 40 nm (Fig. 2III). The diameter of A $\beta$ 42 amorphous aggregates and oligomers in the presence of Cu(II) ions were more than that of A $\beta$ 40 with Cu(II) (Fig. 3IIIA, B). The number of oligomers in the presence of copper ions were significantly higher than A $\beta$ 40 and A $\beta$ 42 samples with no Cu(II) ions. The fluorescence intensities of A $\beta$ 40 and A $\beta$ 42 in the presence of copper ions were significantly lower than those without Cu(II), presumably because they could not have formed considerable numbers of ThT-reactive aggregates (Fig. 1).

#### Aggregation of A $\beta$ in the presence of metal ions and chelators

As it was stated before, there are extensive evidences that link AD pathology to metal dysregulation. It has

been suggested that the problems in the homeostasis of metals in AD patients might be related to amyloid and tau pathologies (Santos et al. 2016; Robert et al. 2015). In this regard, it has been consequently suggested that metal MPACs might have therapeutic potentials in AD. It has also been proposed that metal chelators either might dissolve the deposits in the brain tissue or might prevent amyloid beta aggregation (Santos et al. 2016). It has specifically been reported that CQ can inhibit A $\beta$  accumulations and improve memory capacity in Alzheimer's disease transgenic mice (Robert et al. 2015).

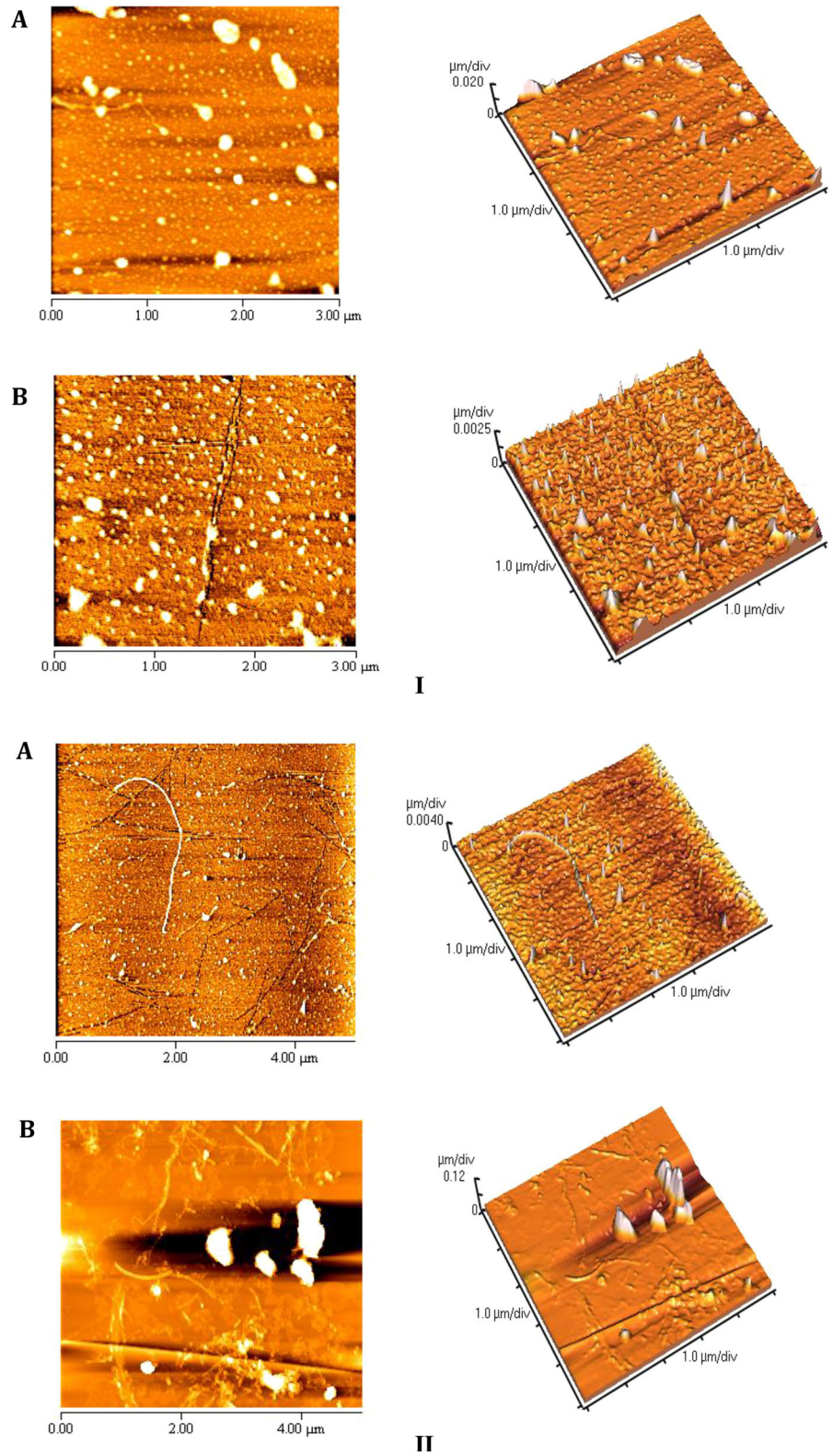
The fluorescence intensities and data from AFM images of A $\beta$ 40 and A $\beta$ 42 in the presence of CQ or EDTA was not significantly different from those of samples containing only A $\beta$ 40 or A $\beta$ 42, and non-fibrillar aggregates were dominantly observed in AFM images (Figs. 1, 3I). Also, the intensities of A $\beta$ 40 and A $\beta$ 42 in the presence of Cu(II) ions and CQ or EDTA was significantly higher than those containing only A $\beta$ 40 or A $\beta$ 42 and Cu(II) ions, which means that both of the chelators could chelate copper ions and alleviate the reduction in intensities that was observed because of the presence of only copper ions (Fig. 1). AFM images of above samples showed that the oligomer contents were reduced (in the presence of CQ), although dispersed clusters and amorphous aggregates of beta-amyloid peptides were also evident (Fig. 3II).

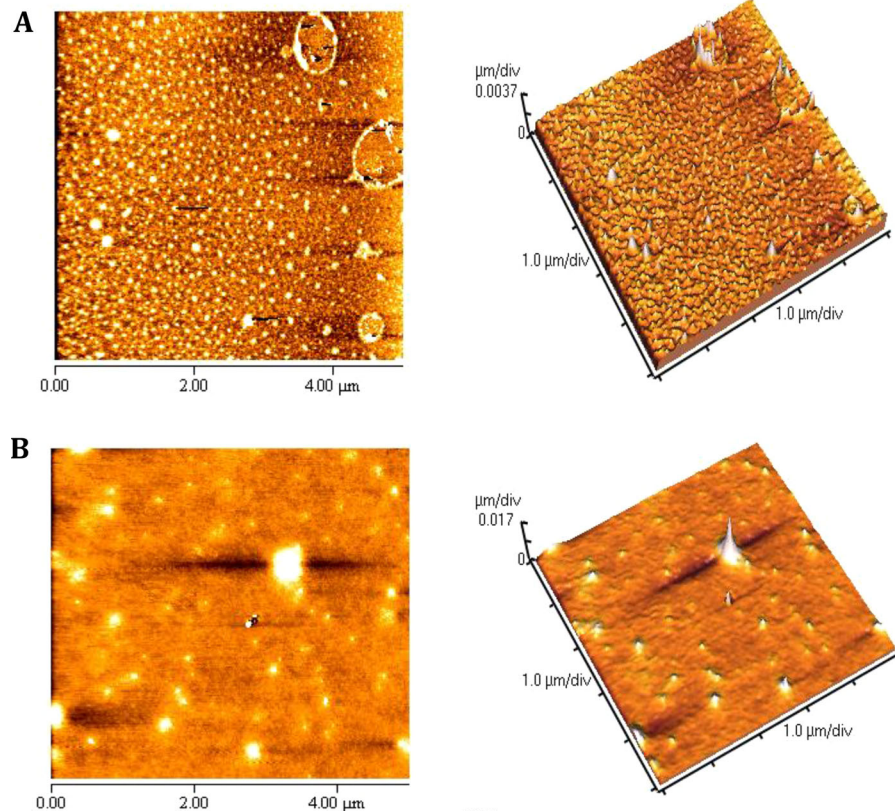
The fluorescence intensities of A $\beta$ 40 and A $\beta$ 42, in the presence of Fe(III) ions containing EDTA, was not significantly different from the sample of amyloids and Fe(III) without EDTA (Fig. 1). AFM images of samples that contained A $\beta$ 40 and Fe(III) ions resulted in the appearance of dense fibrillary aggregates (Fig. 2IIA), changed to the appearance of dispersed fibrillary aggregates after inclusion of EDTA (Fig. 3-III). The fluorescence intensities of the A $\beta$ 40 or A $\beta$ 42 in the presence of iron and CQ (26 and 22 a.u., respectively) was considerably lower than that of only iron ion (37 and 29 a.u., respectively) (Fig. 1). From this observation it can be concluded that CQ is a more effective chelator in reducing the appearance of ThT-reactive species in the presence of iron ion than EDTA.

AFM images of samples containing A $\beta$ 40 or A $\beta$ 42, in the presence of Fe(III) ions and chelators, are presented in Fig. 3III. The presence of EDTA in A $\beta$ 40 + Fe(III) samples resulted in the appearance of numerous fibrillar species (Fig. 3IIIA), which could



**Fig. 2** **(I)** AFM images of aggregates containing: **(I)** Only amyloid peptides; **(II)** Amyloid peptides and Fe(III); **(III)** Amyloid peptides and Cu(II). **A** A $\beta$ 40 and **B** A $\beta$ 42





**Fig. 2** continued

also be seen in samples containing only A $\beta$ 40 + Fe(III) (Fig. 2IIA) and was also the case for A $\beta$ 42 (compare Figs. 2IIB, 3IIIC). How about the observed reductive effect of CQ on the intensities obtained in the presence of either A $\beta$ 40 or A $\beta$ 42 and Fe(III)? Comparison between Fig. 2IIA, B (A $\beta$ 40 and A $\beta$ 42, respectively, in the presence of iron) and Fig. 3IIIB (A $\beta$ 40 + Fe + CQ) and 3IIID (A $\beta$ 42 + Fe + CQ) shows that disappearance of fibrillar species in the presence of CQ (Fig. 3IIIB) affirms the related fluorescence results (Fig. 1). In the case of A $\beta$ 42 (Fig. 3IIID), the fibrillar species still can be observed (compare with Fig. 2IIB) and is not that much quantitative.

## Conclusions

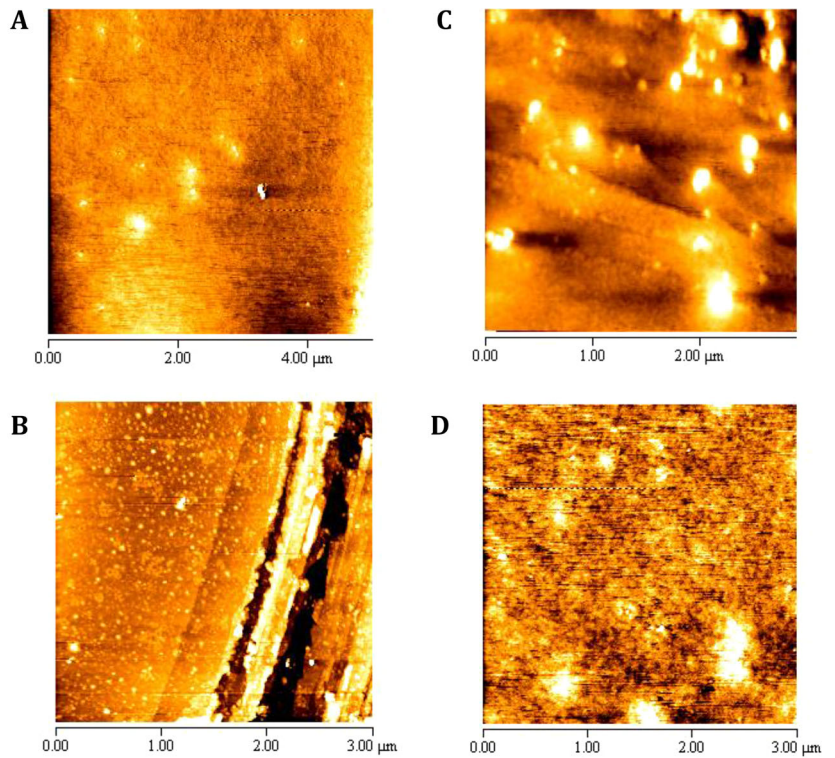
Copper and iron dysregulation is considered as a consequence of aging and there are considerable evidences that is also involved in the pathogenesis of AD as a neurodegenerative disease. The main effect of

## III

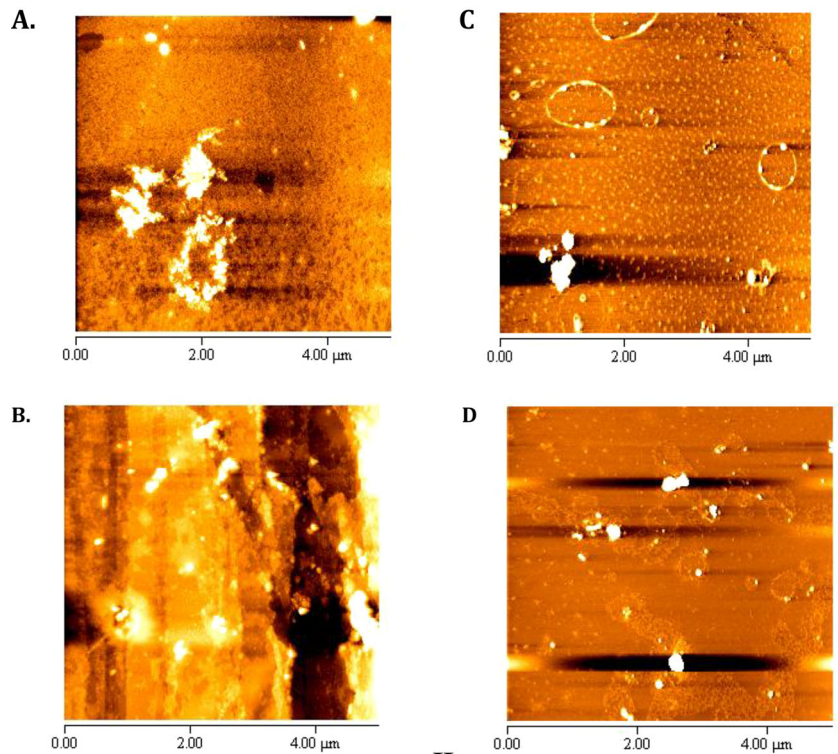
this metal ion dysregulation is proposed to be the oxidative stress and the consequent release of ROS species. Also, these metal ions have been shown to interact with amyloid peptides and potentially affect protein misfolding and aggregation. Our results show that copper not only has no promotive effect in the fabrication ThT-reactive species (i.e. beta sheet structures) but also has a reductive effect. On the other hand, this work shows a considerable positive effect of Fe(III) ions on the appearance of ThT-reactive species and aggregates. Both of these ions were chelated by both of the chelators that were used in this study (EDTA and CQ) and the consequent effect of chelating these ions was observed. In the case of copper, its chelation resulted in an increase in its aggregative properties (copper itself reduced the aggregation), and in the case of iron, only CQ could decrease its positive aggregative effect on both peptides. So according to this study, CQ, which is able to pass the BBB, only shows its potential anti-aggregative effect on Fe(III) ions and this anti-aggregative effect is relevant in the case of Cu(II) ions.



**Fig. 3** **(I)** AFM images of aggregates containing amyloid peptides and chelators: **(IA)** A $\beta$ 40 + EDTA; **(IB)** A $\beta$ 40 + CQ; **(IC)** A $\beta$ 42 + EDTA; **(ID)** A $\beta$ 42 + CQ. **(II)** AFM images of aggregates containing amyloid peptides, chelators and Cu(II) ions: **(IIA)** A $\beta$ 40 + Cu + EDTA; **(IIB)** A $\beta$ 40 + Cu + CQ; **(IIC)** A $\beta$ 42 + Cu + EDTA; **(IID)** A $\beta$ 42 + Cu + CQ. **(III)** AFM images of aggregates containing amyloid peptides, chelators and Fe(III) ions: **(IIIA)** A $\beta$ 40 + Fe + EDTA; **(IIIB)** A $\beta$ 40 + Fe + CQ; **(IIIC)** A $\beta$ 42 + Fe + EDTA; **(IIID)** A $\beta$ 42 + Fe + CQ

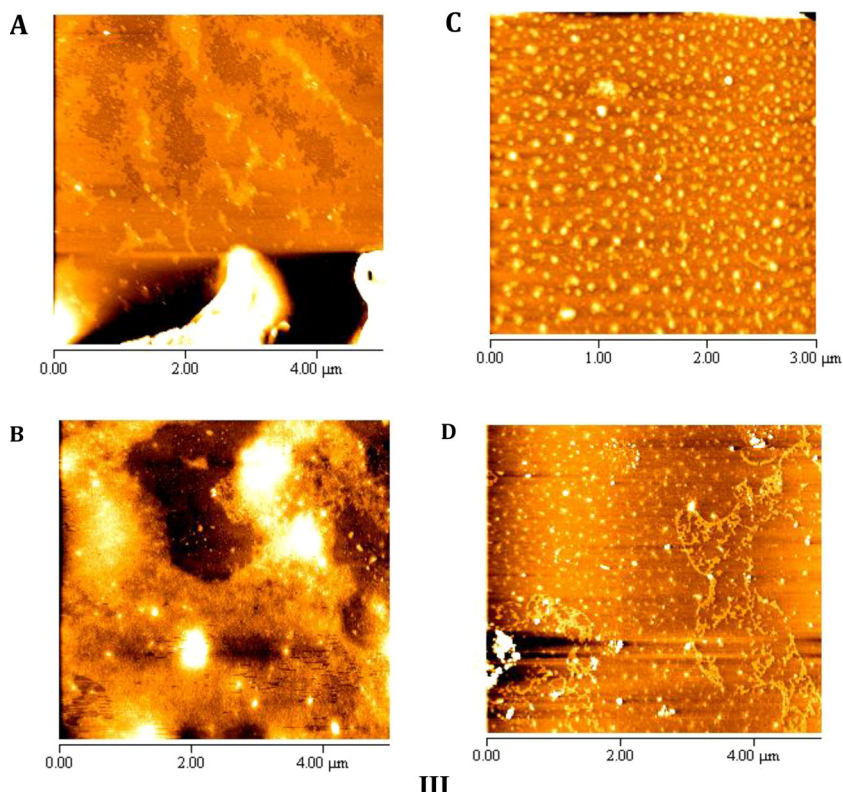


**I**



**II**

Fig. 3 continued



III

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