STUDY OF THE INTERACTION BETWEEN AMYLOID BETA AGGREGATES AND THE LPFFD PEPTIDE BY USING ATOM FORCE MICROSCOPY TECHNICS AND GOLD NANOPARTICLES

Aurora Morales [a], Leonardo Caballero[b,c], Francisco Melo[b,c] and Marcelo J. Kogan[a,b*]

[a]: Departamento de Química Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile. [b]: Centro para la Investigación Interdisciplinaria Avanzada en Ciencias de los Materiales, Av. B. Encalada 2008, Santiago, Chile. [c]: Departamento de Física. Facultad de Ciencias. Universidad de Santiago de Chile, Santiago, Chile

Introduction

The **amyloid beta peptide** (**Aβ**), involved in Alzheimer's disease, change the conformation in pathological conditions forming **toxic aggregates** (**TA**)[1], like **amylospheroids** (**AE**), protofibrils, globular aggregates and **amyloid fibrils** (**AF**), that have a neurodegenerative effect. Different authors have studied and postulated structural models with the disposition of Aβ monomers in the TA.[2] However the disposition of the Aβ molecules in the AF is still unclear. In the present work, we study by force spectroscopy mode the interaction of the TA of Aβ adsorbed on a **Highly Ordered Pyrolytic Graphite (HOPG)** surface with a gold-CLPFFD linked tip. Through this procedure we can recognize TA with structural domains that guide us in the molecular structures of these aggregates.

The force curves give information of the interaction between the AF of Aβ and the peptide sequence CLPFFD. The main interaction observed is the adhesion attributable to the exposed hydrophobic residues in the AF.

In a previous study, the structures of AF were observed using atomic force microscopy (AFM), and AF exhibits a nodular structure with a ~100-nm periodicity nm[3]. In another study, **gold nanoparticles** (**AuNPs**) were linked to the peptide **CLPFFD** that selectively attaches to the AF of Aβ.[4] It is believed that the AuNP-CLPFFD recognize hydrophobic domains of the β-sheet structure with a 100 nm of periodicity.[5]

This would indicate that this periodicity set out groups that allow interaction between AF and L, F and F residues of such peptide.

Moreover, we incubated the AuNP-CLPFFD and TA *in volume* and the formed complex were observed on a Mg^{+2}-Ruby-Mica surface. In this case AuNP-CLPFFD are included into the TA structure periodically. These results indicate that there are periodical domains in the AF.

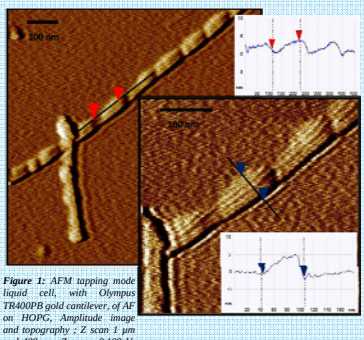


Figure 1: AFM tapping mode liquid cell, with Olympus TR400PB gold cantilever, of AF on HOPC, Amplitude image and topography : Z scan 1 µm and 400 nm; Z range 0.100 V; PF-scanner

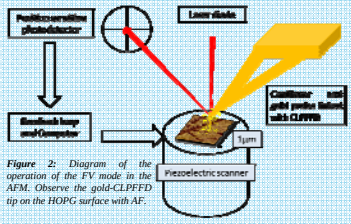


Figure 2: Diagram of the operation of the FV mode in the AFM. Observe the gold-CLPFFD tip on the HOPG surface with AF.

Results

Force Volume (FV) Spectroscopy

This mode of the AFM was used to obtain detailed information about the structural domains in the AF of $A\beta_{1-42}$. The FV spectroscopy can be used after stabilization of the TMAFM and after located the study area. **(Figure 2)**

During the FV, the tip collected information about the magnitude of the interaction between the CLPFFD functionalized probe and the TA on the HOPG surface. Three 3 µm areas were divided in 64 pixels. Each pixel represent a 64 interaction curves average and 47 nm² areas was evaluated. **(Figure 3)**

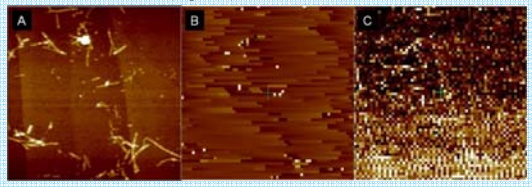
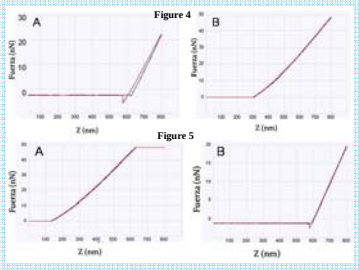


***Figure 3A:** TMAFM in fluid Height image, AF on HOPG; Z scan 3µm , Z range: 30 nm; 512 pixels; Veeco NP Si3N4 probe; J scanner; **3B:** FV Height image, AF on HOPG; Z scan 3µm , Z range: 20 nm; 64 pixels; Olympus TR400PB probe with CLPFFD; J scanner; **3C:** FV image; AF on HOPG; Z scan 3µm , Z range: 10 nm; 64 pixels; Olympus TR400PB probe with CLPFFD; J scanner*

CLPFFD –AF interaction trough FV

The interaction force curves between **gold-CLPFFD** and **AF** adsorbed on HOPG indicated **adhesion forces** (F_a) < 8 nN **(Figure 4A)**, and most of them are of hard contact 0 nN **(Figure 4B)**. This situation repeated in gold on HOPG force curves **(Figure 5A)**. The force curves between gold-CLPFFD and HOPG indicated F_a < 2.5 nN **(Figure 5B)**. Curves in the 6A and 6B figures were acquired using a Novascan PT-GS-AU gold probe, with a 2.5 µm nominal radius microsphere in one extreme, and functionalized with CLPFFD trough the same technique. The F_a value were used for the **adhesion energy** (γ_{SL}) calculation. **(Table 1)**



Methodology

Functionalization of atomic force tip

To study the interaction of the CLPFFD peptide with Aβ, we functionalized a Olympus TR400PB gold cantilever with a tip with 15 nm of nominal radius. This cantilever was introduced for 30 s in a low pressure camera (10^{-2}mbar) that generates a plasma etching stream that can withdraw 2 nm of the metallic surface of the tip. The Langmuir-Blodgett method was used for the functionalization with a $1x10^{-7}$ M of CLPFFD solution, which formed a gold monolayer adsorbed on the surface of all cantilever.[6]

Preparation of short AF and adsorption on HOPG

Aggregates are obtained from 0.05 mg of lyophilized $A\beta_{1-42}$ (r-*Peptide*). This is re-suspended in 1 mM NaOH, and adjust pH to 7 with 10 mM NaOH. The aggregation process was initiated using 20 mM phosphate buffer, pH=7.4, to reach a 100 µM $A\beta_{1-42}$ concentration. The incubation period was 20h/23°C. 30 µL of the TA obtained were allowed to adsorb for 15 min at room temperature on freshly cleaved HOPG (SPI-1), that has a strong affinity for hydrophobic structures [7].

Atomic Force Microscopy (AFM)

All images was performed with a commercial Multimode IIIa atomic force microscope controlled by a Nanoscope electronics 5.30r3.sr3 (Digital Instruments, Santa Barbara, CA), equipped with either a 120 µm J-scanner, or a 12 µm E-scanner, or a 40 µm PF-scanner. The images were taken either in liquid (5 mM buffer phosphate pH 7.4) using a tapping mode (TMAFM) in liquid cell with the O-ring seal, and using either a Veeco NP (TMAFM) or a Olympus TR400PB (FV) probes **(Figure 1)**, or in air using a TMAFM in air without the O-ring seal, and using the Veeco TESPA-7 probe (see the AuNP-CLPFFD experiment).

Incubation of AF with AuNP-CLPFFD

30 µL of 100 µM AF of $A\beta_{1-42}$ solution was prepared, and were mixed in a vial with 1 µL of AuNP-CLPFFD in citrate (2 µM y 6nM respectively). This was incubated at room temperature for 24 hours, and was 5 hours in the magnetic stirrer.

Functionalization of Ruby-Mica surface

1 cm² piece of muscovite ruby mica was glued to a painted steel support. 30 µL of a 9mM Cl_2Mg solution were allowed to adsorb for 10min at room temperature on freshly cleaved ruby mica. The surface was carefully rinsed with nano-pure water and gently dried under a N_2 stream.

Force Curves	Tip Radius (R)	F_a	γ_{SL}
Gold-CLPFFD/ HOPG + AF	~15 nm	<8 nN	0-56.6 µJ/m²
Gold/HOPG	~2.5µm	0	0
Gold-CLPFFD/ HOPG	~2.5µm	<2.5nN	<0.085 µJ/m²

***Comparative table 1:** Tip nominal radius (R); Adhesion force (F_a) and Adhesion energy (γ_{SL}) of the force curves obtained in the FV experiments*

$$\gamma_{SL} = \frac{F_a}{R3\pi}$$

Tapping Mode of Atomic Force Microscopy in air

Immediately before imaging, 30 µL of sample was allowed to adsorb for 15 min at room temperature on the freshly dried Mg^{+2}-Ruby-Mica. The surface was carefully rinsed with phosphate buffer 5 mM, then rinsed with nanopure water and gently dried under a N_2 stream.

The images were taken in the air TMAFM mode with an E-scanner, and using a Veeco TESPA-7 probe. We observed samples with AuNP-CLPFFD **(Figures 6 and 7)** and without them. **(Figure 8 and 9)**

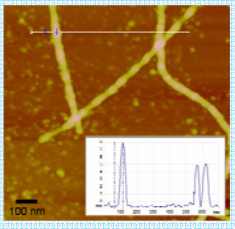


***Figure 6:** Air TMAFM Height image; AF with AuNP-CLPFFD on Mg^{+2}-Ruby-Mica Z scan: 1 µm; Z range: 15 nm; 512 pixels; E scanner. Section: vertical distance 8.646 nm*

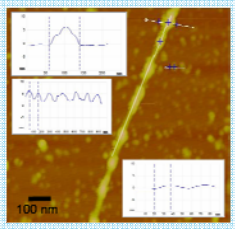


***Figure 7:** Air TMAFM; height image; AF with AuNP-CLPFFD on Mg^{+2}-Ruby-Mica Z scan 1 µm; Z range 15 nm; 512 pixels E scanner; Superior section: horizontal distance 79 nm; Middle section: horizontal distance 101 nm. Low section: vertical distance 1.335 nm*

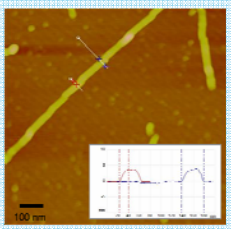


***Figure 8:** TMAFM Height image, AF on Mg^{+2}-Ruby-Mica; Z scan 1 µm; Z range 15 nm; 512 pixels; E scanner; Red section: vertical distance: 3.9 nm; Blue section: horizontal distance: 43 nm*

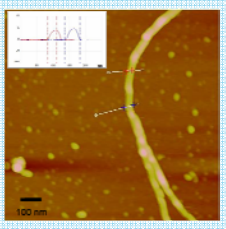


***Figure 9:** TMAFM Height image, AF on Mg^{+2}-Ruby-Mica; Z scan 1 µm; Z range 15 nm; 512 pixels; E scanner; Red section: vertical distance: 3.5 nm; Blue section: horizontal distance: 47 nm*

Histogram

The AuNP-CLPFFD have an average diameter of 8.11 nm with a standard deviation (S.D.) +/- 1.956 nm. But, in the 134 units histogram we can observe 2.2 nm of minimum diameter and 12.6 nm of maximum diameter size. In the **Tables 2 and 3** we present statistics from the AF topography with and without AuNP-CLPFFD and vertical/horizontal relation value. The amyloespheroids (AE) are present in all images and can be confused with AuNP-CLPFFD, but they have difference in size. (**Table 4**)

Table 3 AF studied	Vertical Low distance (X)	Horizontal Low distance (X)	Relation
With AuNP-CLPFFD	4.95 nm (S.D. 1.0)	44.02 nm (S.D. 6.9)	0.13 (S.D. 0.79)
Without AuNP-CLPFFD	4.853nm (S.D. 0.8)	41.2 nm (S.D. 4.0)	0.11 (S.D. 1.06)

Table 4 AE studied	Height	Diameter	Relation
With AuNP-CLPFFD	1.66 nm (S.D. 0.8)	35.56 nm (S.D. 8.7)	0.04 (S.D. 0.014)
Without AuNP-CLPFFD	2.0 nm (S.D. 1.9)	31.53 nm (S.D. 7.4)	0.05 (S.D. 0.03)

Table 2 AF studied	Vertical High distance (X)	Horizontal high distance (X)	Relation
With AuNP-CLPFFD	6.19 nm (S.D. 0.6)	47.78 nm (S.D. 6.0)	0.13 (S.D. 0.79)
Without AuNP-CLPFFD	5.7 nm (S.D. 1.1)	42.6 nm (S.D. 4.7)	0.13 (S.D. 0.018)

Discussion

•The AF that have a 100 nm of periodicity present a compatible topography with the recent models about their molecular structure and indicates that this AF adopt a helical conformations to protect from the water the hydrophobic domains (**Figure 1**). In the FV experiments we could not find a correlation between the height image during the FV and the FV image. To obtain information about the periodicity, more resolution FV is necessary and this is not available in the execution optimum time **(Figure 3).** The F_a between gold probe and HOPG surface is 0; therefore, all other interactions observed correspond a CLPFFD and HOPG or TA interactions. The hydrophobic interaction between CLPFFD and HOPG surface is <0.085 µJ/m². This is lower than the typical hydrophobic interactions (50 µJ/m²).[9] The CLPFFD and AF interactions is among 0 and 56.6 µJ/m² (**Table 1**). That indicates a lower or non existent hydrophobic interaction which could be explained by the high TA adsorption on the HOPG through the hydrophobic domains in the AF. In this way, the AF on the HOPG surface can't expose their hydrophobic residues, which difficult the interaction with **CLPFFD**.[5]

•The main idea of use the AuNP-CLPFFD in the inhibition of the $A\beta_{1-42}$ aggregation process is the possibility of this LPFFD sequence to be linked on the hydrophobic sequence (^{17}LVFFA^{21}) of $A\beta_{1-42}$. This hydrophobic interaction can occur when we mixed AuNP-CLPFFD with AF of $A\beta_{1-42}$ for a few hours. In the Mg^{+2} ruby mica substrate the TA, are attached to the surface by charge-charge interactions and the structure of the complex $A\beta_{1-42}$/AuNP-CLPFF is modified with respect to $A\beta_{1-42}$. We make statistics about the size of the AF with and without the AuNP-CLPFFD on Mg^{+2}ruby mica substrate in air TMAFM (**Figures 6, 7, 8 and 9**). The AF has two sites where we can measure horizontal (diameter) and vertical (high) distance, and this is a superior and lower zone of the AF, like we can see in the superior topography in the **figure 1**. (**Tables 2 and 3**); a difference between the AF diameter with AuNP-CLPFFD and without them. Other authors have explained that NP can form pores and lowering the integrity of lipid membranes; and the NP can be enveloped for the membranes resulting in a curvatures outside of that region .[10] In the case of AF in presence of AuNP-CLPFFD are observed a 100 nm periodic inclusion (**Figure 7**), which reveals that in the fibrils are exposed periodical hydrophobic domains that allow the interaction of FA with AuNP-CLPFFD.

Bibliography

[1]Pike C.J. *J Neuroscience*. 1993;13:1676–87
[2]Sato T. *Biochemistry.2006, 45, 5503-5516*
[3]Arimon M. *FASEB J.* 2005;19(10):1344-6
[4]Soto C. *Nature Medicine*. 1998;4(7):822-6
[5]Kogan M.J. *Nano Letters*. 2006;6(1):110-5
[6]Duschl C. *Biophysical J.* 1994;67(3):1229-37
[7] Morris VJ. Londres, ICP, 1999, 332 p.
[9]Helm C. *Biochemistry*.1992; 18;31(6):1794-805
[10]Roiter Y. *Nano Letters*. 2008;8(3):941-4

Acknowledgement

FONDECYT 1061142 and FONDAP 11980002 (17 07 002), projects