Aggregation of Alzheimer’s Amyloid Fibrils Studied with Atomic Force Microscopy

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Abstract

The aggregation and malformation of the Amyloid beta peptide Aβ(1−40) is strongly believed to be involved in the development of Alzheimer’s disease (AD). With this thesis we hope to provide important clues to further understanding of amyloid formation in vitro, near physiological conditions. We use the Atomic Force Microscope (AFM) which provides three-dimensional images with vertical resolution down to Angstroms. Together with a previously developed add on tapping mode system we investigate the aggregation paths for Aβ(1−40) in three different relevant physiological buffer solutions. The AFM system is extended with an external data acquisition system for increased image resolution. Our results show that protein aggregation can be very complex, many different intermediates with a large variety of morphologies are observed, including protofibrils and large mature fibrils. Our results also indicate that the appearance of protofibrils, i.e. an intermediate stage in the fibrillization, depends on the peptide concentration. It is also shown that pretreatment of samples are very important, there are large differences in aggregation behaviour between a freshly prepared Aβ(1−40) sample and a sample that has been frozen prior to experiments, due to a degradation of peptide concentration. The addition of sodium chloride to the sample reveals an increased aggregation process, fibrils were seen two days earlier than for Aβ(1−40). Also shown is that too large amounts of such [Cl]− ions in the buffer solution rearranges the fibrils structure into amorphous aggregates. Aluminium has for many years been suggested as a possible cause in the development of AD. The addition of an aluminium citrate, stable at relevant pH, shows that the aggregation process towards fibrillization is increased, and these fibrils show no tendency of instability. The mature fibrils in the case of added aluminium citrate have larger heights than those observed in freshly prepared and frozen samples. This result may be important as a clue to explain AD pathogenesis, since similar fibrils previously have been shown to be toxic to neurons.
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Chapter 1

Scanning Probe Microscopy

Scanning Probe Microscopy (SPM) covers many related technologies for sample imaging and sample measurements. To mention a few of them, Scanning Tunnelling Microscope (STM) which measures the tunnelling current between tip and sample, the Atomic Force Microscope (AFM) which measures the tip-sample interaction forces, the Magnetic Scanning Microscope (MSM) which measures the strength, polarity and magnetic fields of the sample of interest.

In this chapter the characteristic parts of the Atomic Force Microscope are discussed. Different modes of operation, force-distance curve and also the tip-sample interactions that the AFM operate by will be explained.

1.1 Introduction to Atomic Force Microscope

The development of the Scanning Tunneling Microscope by Gerd Binnig and Heinrich Rohrer is truly a great achievement in the field of physics. They were awarded the Nobel Prize in Physics 1986 for the invention of the STM and shared the prize together with Ernst Ruska (The designer of the first conventional electron microscope) [1].

STM utilizes the tunneling current to obtain images. In order to have a tunneling current the samples must be conducting, which obviously leads to certain restrictions. The Atomic Force Microscope (AFM) is a further development of the latter and with this technique there are no limitations regarding non-conducting samples. One of the advantages of working with AFM is the application to view biological samples such as DNA and proteins. Other sample properties that can be analyzed are friction, electrostatic, chemical and magnetic properties, adhesion, hardness and forces between molecules. When imaging samples with AFM it can provide height information with resolution down to Angstroms.
1.1.1 Principles of Atomic Force Microscope

The principles on how the AFM works are simple. The sample of interest is mounted on the piezo tube scanner. An atomically sharp tip is attached to a reflective cantilever which is held by a larger stationary console. A diode laser beam is focused on the edge of the reflective cantilever. As the scan proceeds in a raster pattern the cantilever deflects along with the surface, reflecting the laser beam into a two element photodiode which detects the movements of the beam. A deflection signal is produced by measuring the difference between the two elements A and B. The deflections signal is being used as an error signal for the computer feedback or directly as an indication of height changes. The setpoint signal allows the user to manually adjust for how much cantilever deflection should be allowed. The feedback in the control unit is working to maintain the cantilever deflection signal constant by adjusting the z-piezo voltage. As the scan proceeds the analogue signals to the piezo tube scanner are being converted by the computer control unit into position and height readings and generated as pictures.

**Figure 1.1.** A schematic of the AFM system. The reflection from the cantilever is captured by the two element photodetector A and B. The differences in light intensities between A and B are measured and a deflection signal is produced. The feedback will attempt to keep the deflections signal constant by adjusting the scanner z-voltage. The Control unit interprete the voltage as height information of the sample topography and produce the image.
1.2 Force interactions

There are many forces interacting between the tip and sample surface. The forces that may occur are all depending on the sample and tip properties and the separation between them. These forces can be traced back to one of the four fundamental forces, the electromagnetic force.

A frequently used model for describing interactions between particles is the empirical Lennard-Jones potential [1]:

\[
U(R) = 4\varepsilon \left[ \left( \frac{\sigma}{R} \right)^{12} - \left( \frac{\sigma}{R} \right)^{6} \right]
\]

[1]

The Lennard-Jones model describes the potential interaction between atoms, where \( \sigma \) and \( \varepsilon \) have dimensions of [length] and [energy]. \( R \) describes the separation distance between atoms. The force between individual atoms is the negative of the gradient of the potential, so we differentiate with respect to \( R \), which yields:

\[
F(R) = -24\varepsilon \left[ 2 \left( \frac{\sigma^{12}}{R^{13}} \right) - \left( \frac{\sigma^{6}}{R^{7}} \right) \right]
\]

[2]

The repulsive forces at very small distances are represented by the first term in [2], and the second term models the attractive forces at large distances.
The interactions can be grouped into, short range interactions and long range interactions. When the tip is brought closer to the sample the long range forces starts to act between tip and sample. The long range forces are mainly attractive interactions, meaning that they will bend the cantilever towards the surface. These long range forces accounts for many different phenomena such as capillary forces, were an adsorbed gas layer on the sample attracts the tip towards the surface, magnetic and electrostatic forces between regions of charge on both surfaces [4].

When the tip continues its approach towards the surface it begins to experience the short range forces, these forces can be both repulsive and attractive. When there are now chemical interactions the short range forces are repulsive, due to the forces between electrons, which obey the Pauli principle, stating that no two electrons in an atom can have identical quantum numbers. Consequently, this interaction will raise the electrons up to a higher energy level, causing the tip to bend upwards.

For further reading on force interactions in SPM, see [1].
1.3 Modes of operation

There are a many different operational modes available depending on what sample surfaces one wish to investigate. Contact mode (CM), Non-contact mode, Tapping mode (TM), Force Volume (FV), Pulsed force mode (PFM) [3].

In *Contact mode* the tip scans the surface in close proximity with the surface. This method is the one explained 1.1. In this method the deflection of the cantilever is measured and compared to a user set deflection. If the two values comes with a disparity, the feedback loop will apply a voltage to the z-piezo to either raise or lower the sample. This method is also called *Constant force*. In addition, the AFM can be operated in *Constant height* mode, where in this mode the feedback system is disconnected and one use the cantilever deflection directly to generate the images. *Constant height* mode is mostly used for imaging flat surfaces with high resolution.

In *Non-contact* mode the tip is situated above the sample surface. The cantilever must be oscillated above the sample surface at such a distance that it no longer is in the repulsive area. This is a very difficult mode to operate in under ambient conditions. Water contamination on tip and sample forms capillary bridges and forces the the tip to jump into contact, disturbing the interaction [4].

*Force Volume* (FV) is used when recording force-interaction images. The cantilever deflection is measured as the tip moves vertically toward the sample until it touches the surface, and then retracts before moving to the next pixel. Force curve measurement allows surface evaluations such as repulsive, attractive and adhesive interactions. In this mode the lateral forces are drastically minimized. Lateral forces comes from when the tip is dragged onto the surface [5].

*Tapping mode* is a technique that allows high resolution imaging of sensitive samples that are easily damaged. The vibrating of the cantilever allows the tip to gently tap the surface of interest. The short time over which the tip is contact means that the tip is coming in contact with the sample in the vertical direction, reducing the lateral forces dramatically, which is the key to not damage sensitive samples. The idea is to oscillate the tip near its resonance frequency. As the sample approaches the tip, the amplitude of the vibrating cantilever is decreased, when withdrawn the amplitude is increased. The feedback system is now set to regulate the z-piezo voltage to maintain constant amplitude of the vibrating cantilever. Images are generated from the changes in z-piezo voltage [5].

*Pulsed force mode* (PFM) was introduced in 1997 belonging to the dynamic force mode family. The idea is to use a continuous approach-retract-cycle as in FV with the exception, the use of an oscillating cantilever. PFM has the ability to image topography, adhesion and surface stiffness. For further reading on PFM see [3].
1.4 The AFM force-distance curve

In addition to topographic images the AFM can provide additional information on the sample properties such as adhesion, bonding forces and hardness of the surface. During the process of scanning the surface the AFM has the ability to measure and record the small forces felt by the cantilever. For small displacements the force on the cantilever is obtained by converting the cantilever displacement into force according to the relationship given by Hook’s law [3]:

\[ F = k (z - z_0) \]  

where \( F \) is the force in Newton (N), \( z - z_0 \) is the deflection of the cantilever in meters (m) and \( k \) the spring constant for the cantilever in N/m. A typical force curve is shown in figure 1.3. From point B to point C there is a linear relationship between cantilever deflection and displacement which indicates that the surface is rigid. If the force curve had been done on a soft sample the relationship would not be linear due to sample deformation.

![Figure 1.3. The figure shows a typical force-distance curve. The cantilever starts at point A, not touching the surface. In this region it starts to feel the attractive long-range forces and if they are sufficiently strong the tip will snap into surface contact at B. Once tip is in contact with the surface the cantilever will deflect more and more until it reaches the desired setpoint value at C, were the process is reversed. When the tip is withdrawn from the sample, adhesion and bonds formed when in contact may cause the tip to adhere to the surface even past the distance from initial snap in D. At E the adhesion force is broken and the tip is clear from the surface [4,6].](image)
Chapter 2

AFM image acquisition

The Nanoscope II developed by Veeco (formerly Digital Instrument) [7], a combined STM and AFM microscope is used throughout this work. Although the microscope is capable of presenting high resolution topographic images the Nanoscope II, as most AFM:s has it's limitations, it can only provide images with a maximum image resolution of 400×400 pixels. This chapter describes the method of increasing the image resolution in one direction for the available Nanoscope II. An increased image resolution will be helpful with the detailed structural understanding of the peptide Aβ(1–40).

2.1 Data acquisition system

As mentioned before the Nanoscope II comes with a maximally image resolution of 400×400 pixels. To increase the image resolution one would have to sample the analogue signals from the piezo tube scanner faster. The original software which controls the scan has a limit of 400 scanlines. To be able to increase the image resolution in one direction we increase the sampling rate for every scanline. Due to copyrights on the Nanoscope II there is need to acquire the analogue signals through a external PC, and thus generate the images. The faster sampling that is required is done by a National Instruments multifunction data acquisition hardware (DAQ), model PCI-6110. The DAQ has the following properties:

4 analog input Channels 5 Ms/s per Channel, 12 bit resolution, Maximum range ± 50 V, Minimum range ± 200 mV and bandwidth 5 MHz [8].

Matlab was chosen as platform because of it’s compatibility with the DAQ and also with aspects for user convenience by means of a Graphical User Interfaces, (GUI’s) The signals that we acquire trough the DAQ are streamed to disk via Matlabs Analog Input (AI), at a maximum rate of 5 MS/s.
2.1.1 Data acquisition hardware setup

The Maximum and Minimum range of the hardware analogue input is ± 50 V. The piezo-scanner is controlled by ± 220 V. To meet the requirements of the DAQ we have to dampen these signals before acquiring them into the external PC. This is done by running the signals through a 10 MΩ resistor. There are four channels available on the DAQ, which have been assigned accordingly:

Channel 0 = X-piezo signal, Channel 1 = Y-piezo signal, Channel 2 = Z-piezo signal, Channel 3 = Deflection signal

During a scan the x, y, z -piezo signals will vary quite a lot. Since Matlab uses 12 Bit precision we want to record the analogue signals as narrow as possible i.e. to minimize possible disturbances. The x-signal will depend on three things, the scansize, the offset and which piezoelectric scanner one uses. The z-signal is depending on the sample topography and the incline of the scanner head. The y-signal will have a maximum and a minimum in one image. Since we are only concerned with increasing the number of pixels in the x-direction there are two things that we need to know in order to set the right samplerate. We need the ongoing scanrate and the wanted image resolution. Because the external PC doesn’t have any information on have the ongoing scan is proceeding, we need to record both trace and retrace. These can later be used to form two different images depending on scandirection. To achieve the desired image resolution the sampling rate has to be set according:

\[ F_s = 2 f_s I_r \]  

where \( F_s \) is sample rate (Hz), \( f_s \) the ongoing scan rate (Hz) and \( I_r \) is the desired image resolution.

2.1.2 AFM acquisition program

The program that has been developed is easy to use with “push buttons”, “edit text boxes” and “pop-up menus”. The program mainly uses four functions to generate images. These are explained further below. The Matlab based program can be viewed in figure 2.1.

- The Testscan function uses a pre-scan routine that records 5000 samples on all channels and determines the ongoing scanrate and finds the channel range of our analogue signals. The scanrate is determined by finding two adjacent maxima from the x-signal triangular wave. The z-signal range we want to set manually so we only need to know maximum and minimum of the signal. While running the Testscan function you can chose to plot any of the signals to visually see if they are correct.
• **Start / Stop** function activates the simultaneous recording of all four channels. All data is streamed to disk via Matlabs AI and saved as a .daq file.

• The **Split** function sorts all data. This might seem easy but there are a number of problems to overcome before an image can be viewed. The procedure involves filtering of the signals, sorting the data into consecutive trace and retrace lines. While filtering the signals and removing possible disturbances there is a big risk that the recorded lines will end up being different in length. Since Matlab requires arrays to be equal in length we needed to sort and shorten our arrays after the smallest amount of elements (pixels) in the trace and retrace. The result from this procedure is a matrix of $400 \times$ desired resolution.

• **Plot** function plots the image using a colormap.

---

**Figure 2.1.** The figure show the AFM acquisition program, were the main functions are Testscan, Start/Stop, Split and Plot. The plot shows the changes of the z-signal i.e the raise and lowering of the sample.
Chapter 3

Alzheimer’s Disease

This chapter is intended to give a general overview of Alzheimer’s disease (AD). The cause of the disease in present situation is not yet known. Research done on brains with AD shows an overwhelming quantity of beta-amyloid plaques and neurofibrillary tangles. These are believed to be of great importance in the development of AD. There will be a description on how beta-amyloid plaques are formed, and an explanation for neurofibrillary tangles. Also in this chapter we present a proposed model for the amyloid aggregation path and fibril structure.

3.1 General overview of Alzheimer’s disease

Alzheimer’s disease in present situation account’s for 50%–60% of all dementia patients aged 65 and older. Dementia is a collective name for a progressive brain dysfunction which gradually increases and cannot be reversed. It slowly progresses and affects memory, language, the capacity of thinking and often leads to restrictions in the daily activities which used to be routine [9].

The brain is made up of billions of neurons. They can be divided into many different groups, each group having a specific job to perform. For example, one group may be in control of the thinking process, where another group controls learning capabilities. Others may be responsible for muscle stimulation, ability to walk etc. The ability to keep neurons healthy is depending on three processes, communication, metabolism and repair. AD is totally devastating because it interrupts all three processes.

3.2 Amyloid plaques

Amyloid plaques are mainly built up by a protein called beta-amyloid, a protein fragment clipped from a larger protein called amyloid precursor protein (APP) see figure 3.1. APP is protein found in most cell membranes naturally throughout the body. There are two possible functions suggested for APP, regulation of the proteases activity and secretion of soluble beta-amyloids. The plaques themselves are to be
found outside between neurons mostly in the hippocampus and cerebral cortex, where they form insoluble clusters which somehow are highly toxic to healthy neurons.

The beta-amyloid fragment is found in varies lengths between 39 and 43 residues, where a residue corresponds to an amino acid. The two most common fragments are Aβ(1–40) and Aβ(1–42). In senile plaques as much as 90% of total amount of fragments are Aβ(1–40), 10% are fragments containing Aβ(1–39,42,43) [9]. The protein fragment aggregates through a multi-complex reaction, where there are many different structures seen, ending up in what’s called amyloid fibrils, which come in numerous numbers of morphologies.

![Figure 3.1](image1.png)  
**Figure 3.1.** The figure shows the formation of beta-amyloid where enzymes acts on the APP molecule and clips it into small fragments, one of them being beta-amyloid [4,10].

Once the beta-amyloid fragments are formed they begin to form tangles outside the cell, and together with other molecules and non-nerve cell they form the insoluble structure called amyloid plaques.

![Figure 3.2](image2.png)  
**Figure 3.2.** The figure shows the formation of beta-amyloid fragments. The beta amyloid forms with other molecules and non-nerve cells the insoluble structure called amyloid plaque [4,10].
3.2.1 Neurofibrillary tangles

The connection between healthy neurons inside the brain are made by axons. Inside the axons there are a support structure called microtubules, which acts like transport guideline for molecules and nutrients into and from the cell. The microtubules structure is stabilized with a special protein called Tau.

![Figure 3.3](image)

**Figure 3.3.** The figure shows a healthy neuron with it’s supporting microtubules structure. The microtubules are stabilized with the Tau protein [10].

In AD, Tau changes chemically and starts to detach from the microtubules and connect to each other into neurofibrillary tangles. Once detached, the microtubules gets less stable and finally disintegrates destroying the neurons transport system [10].

![Figure 3.4](image)

**Figure 3.4.** The figure shows a diseased neuron. The Tau protein has changed chemically and detached from the microtubules chain and formed tangled clumps. The neuron starts to disintegrate and finally dies [10].
### 3.3 Amyloid-beta peptide

As mentioned earlier the beta amyloid peptide is derived from the beta amyloid precursor protein APP. The APP comes in three major isoforms, APP\(^{695}\), APP\(^{751}\) and APP\(^{770}\), where APP\(^{695}\) exclusively is found in neurons. The other isoforms are found in both neural and non-neural cells. The APP is processed by three enzymes named β, γ and α-secretases. Depending on which one of the three enzymes acting on the APP and at which terminal, different fragments are produced.

**Figure 3.5.** Schematic overview of the APP cleavage. A combination of β-secretase and γ-secretase snips the APP protein and produces the AB-peptide. Image from http://www-ermm.cbcu.cam.ac.uk/0200501Xa.pdf [11].

Experimental evidence suggests a combination of the two enzymes β and γ for the cleavage and formation of beta amyloid fragments. The β-secretase cleaves APP somewhere between residues Met\(^{671}\) and Asp\(^{672}\) producing sAAPβ and C99 fragments, and together with γ cleavage to C-terminal at either Val\(^{711}\) or Ile\(^{713}\) two different fragments are produced, the shorter peptide Aβ\((1−40)\) or the longer Aβ\((1−42)\).

There are also genetic mutations within the APP molecule to take in consideration. These mutations give rise to different mutations of the Aβ peptide. For further reading see [4,10].

### 3.4 Structure and aggregation paths of Amyloid beta peptides

Proteins play a key role in almost all biological processes, and they mediate a wide range of functions such as coordinated motions, immune protection and growth. The building blocks of a protein are the amino acids, and the sequence of the amino acids determines the protein structure and functionality. For a protein of a certain kind to work properly it has to be folded correctly. If it’s not, it cannot carry out it’s particular task, but even worse, sometimes an incorrectly folded protein starts to poison the cells around it, which presumable is the case in AD.
Amyloid formation is a process where a normal protein changes its structural formation and in some cases becomes toxic to neurons. Experiments have shown that the Amyloid-beta peptide has the ability to form a number of different aggregates such as oligomeric species, different amorphous conformations, protofibrils or protofilaments with various lengths, spherical particles of different magnitudes and large mature fibrils [12,13,14]. Which of the different conformations that are formed and how fast the aggregation progresses depends on many things such as peptide concentration, pH, temperature, salt concentration [4,15,16].

There are no exact models that predict the different aggregations paths for Aβ peptide although many have been suggested. A recent proposed aggregation pathway states that the monomeric form of Aβ peptide is found in two different forms relevant to aggregation. One form is the unfolded state which has no ordered structure and the second one is a folded state rich in β-sheet conformations prone to fibril formations. It is suggested that the first form relates to normal physiological conditions and the latter in the disease. The monomers in the first form are involved with the building of spherical particles that reach a critical size of stability, hence a predisposition towards amorphous aggregates which may be degraded by proteasas. For the process to go in the direction of fibril formation the aggregation pathway must reach a critical concentration of folded peptide, readily protofibrils, which by cooperative association assembles into larger mature amyloid fibrils [17].

The hydropathy is very important for how a protein folds, but there are no exact ways to now how a given residue belonging to a certain chain react in a favourable way, is it located in the non-aqueous interior or on the solvent exposed surface?. A proposed structural model, see figure 3.6, shows the folding for Aβ(1−40) protofibril/protofilaments. This model predicts how the hydrophobic interactions are maximized within the environment of cross-β structure, also how it can avoid the unfavourable electrostatic interactions within the fibril/protofilamentus core. For further reading see [18].

Figure 3.6. The figure shows a structural minimal model of AB (1-40) protofilament with fibril long axis pointing out from the page, based primarily on solid-state NMR, also consistent with restrictions from EM, X-ray scattering, EPR and biochemical techniques. (a) shows a diagram of residues 9-40, where there are two B-strands per molecule and two B-sheets. (b) shows the atomic representation where the sidechains are colorcoded according: green=hydrophobic, magenta=polar, red=negatively charged, blue=positively charged. Images from [19].
Chapter 4

Results and Analysis

In this chapter we present the results of direct observations of the performed experiments which was carried out with a previously developed AFM-add on Tapping mode (TM) system [4]. There will be a detailed description on how the peptides were mixed and prepared before they were put into the AFM microscope and viewed, also a description on how the samples have been investigated. Images obtained with the developed external image acquisition program will also be presented.

4.1 Experimental object

The purpose of these experiments is to view, document and analyse the aggregation paths and characterize the different fibril morphologies for the peptide wild type Aβ(1−40). The experiments are carried out in three different relevant physiological buffers, the first being a standard buffer containing TRIS, EDTA, KCL and NaN3. In the second buffer we add NaCl to better mimic real physiological conditions, where the salt concentration in the human body being 140 mM. In the third buffer we add aluminium-citrate Al₃(OH)(−HCit)₃⁴⁻ to the standard buffer solution. The reason for adding an aluminium citrate is that there were discussions as far back as the 80th about its influence in Alzheimer’s disease. To date the common notion is shared that aluminium cannot be responsible for AD by itself, but it cannot be discarded as a contributing factor. It has been suggested a number of ways for a aluminium to end up in the human body [20,21]. The aluminium complex that we use in our experiments has been shown to be the major species among other Al-citrate complexes at pH values of 7.4 see figure 4.1.
4.2 Material and methods

The peptide Aβ(1−40) that was used during the experiments were synthesized using solid phase fluorenylmethoxycarbonyl (FMOC) synthesis and purified (HPLC, in water/acetonitrile gradient) by O. Antzutkin [22]. A physiological relevant buffer solution was prepared containing TRIS 10 mM, EDTA 0,5 mM, KCL 10 mM, NaN3 0,01 w/o, the buffer was adjusted with 550 µl HCL from pH 8.8 to pH 7.4. The peptides were weighted and dissolved in buffer solution to a concentration of 50 µM and put in 12 standard plastic tubes a’ 190 µL. All samples were instantly frozen with liquid nitrogen and finally stored in −70ºC.

Four different experiments were performed:

- Wild type Aβ(1−40) solution with concentration 50 µM, initially frozen.
- Wild type Aβ(1−40) solution with concentration of 50 µM, initially frozen together with aluminium complex, concentration150 µM, added after the thawing of the sample tube.
- Wild type Aβ(1−40) solution with concentration of 50 µM, initially frozen together with Sodium Chloride added at concentration 140 mM.
- Wild type Aβ(1−40) solution with concentration 50 µM freshly prepared, i.e. without initial freezing with liquid nitrogen.

Before imaging, the samples were prepared by placing 15 µL of peptide solution onto a freshly cleaved mica and left to incubate in a small box for 15 minutes. After incubation the sample was rinsed with 3×15 µL buffer clearing any unbound protein.
Before mounting the sample into the microscope the fluid-cell was flushed with buffer solution. After the sample had been inserted into the microscope additional buffer was added, usually 50 µL.

Images were obtained under ambient conditions with a modified [4] Nanoscope II scanning probe microscope (Digital Instruments, Santa Barbara, CA) and through the developed AFM acquisition program, see chapter 3. IGOR Pro 4.07 (Wave metrics, Lake Oswego, OR) where used with procedures developed in our laboratory to semi-automatically extract volume, statistics and height distributions. For further reading see [23]. A type D scanner was used throughout the experiments. Short narrow legged silicon nitride cantilevers (spring constant 0.32 N/m) were used except in the last experiment where the silicon nitride cantilevers were oxygen sharpened. Best imaging results were obtained with tapping frequency range 10−11 kHz. The drive amplitude for the cantilever was in the range 100−150 mV and scan rates were usually between 1−2 Hz. For the original software the image resolution were 400×400 pixels. For the newly developed system the desired image resolution should be unlimited in one direction, but insufficient software memory limited the resolution to a maximum of 400×5000 pixels.
4.3 Images obtained with the developed AFM image acquisition program

The panels displayed below shows the images captured with the developed external AFM image acquisition program. Panel A shows small aggregates of wild type Aβ(1−40) frozen. Panel B (48 days) shows mature fibrils of wild type Aβ(1−40) with added aluminium complex.

Figure 4.2. Panel A shows the aggregation of frozen wild type Aβ(1−40) with image resolution 400×3000 pixels, scansize 2µm. Panel B shows the mature fibrils of wild type Aβ(1−40) with added aluminium complex, image resolution 400×2000 pixels, scansize 1.5µm. Images obtained with the developed external image acquisition program. Brighter color corresponds to larger heights.
4.4 Aggregation process for frozen Aβ (1–40)wt

The panels displayed below shows the aggregation process over time. In all the panels the height and length of aggregate/fibril are measured. Panel **A** (26h) after onset shows a few larger aggregates in the range 10−12 nm. A major part of the aggregates in range 1.5−4 nm, no signs of any ordered structure. Panel **B** (48h), a major part of the aggregates in range 2−4 nm. Panel **C** (74h), clearly shows ordered structures. Many protofibrils/protofilaments with lengths between 50−100 nm and heights of 2−6 nm, a few larger aggregates present. Panel **D** (120h) after onset, no signs of any protofibrils, only a small increase in growth of aggregates. In panel **E** (146h), two large groups, one group ranging 4–7 nm the other 7–11 nm. In panel **F** (170h) after onset, a large number of fibrils and aggregates with heights ranging 2–5 nm, fibril lengths 20–250 nm, also larger aggregates ranging between 6–10 nm. Panel **G** (44d) shows a mature fibril.

![Image](image1.png)

**Figure 4.3.** Panels A-G show tapping mode AFM images of the aggregation process for wild type Aβ(1–40) frozen. All images are colour coded, brighter colour corresponds to larger heights.
4.4.1 Probability histograms of aggregate heights for frozen Aβ (1–40)wt

Figure 4.4. Histograms B-F shows the probability and particle distribution of aggregate/fibril for frozen wild type Aβ(1–40).
4.4.2 Fibril characterization for frozen Aβ (1−40)wt

Panel A (day 44) displayed below shows together with the belonging topographic diagram a coiled fibril with maximum height 9.2 ± 0.5 nm.

Figure 4.5. Panel A with corresponding height contour diagram shows the fibril characterization of wild type Aβ(1−40) frozen. Image is colour coded, brighter colour corresponds to larger heights. The scales in the height contour diagrams are, vertically (nm) and horizontally (µm).
4.5 Aggregation process for Aβ (1−40)wt with the added Sodium Chloride

The panels displayed below shows the aggregation process over time. In all the panels the height and length of aggregate/fibril are measured. Panel A (26h) shows no ordered structure, only an even distribution of small aggregates are seen across the mica surface. A large part of the aggregates are in the range 2−7 nm, also aggregates between 7−15 nm. Panel B (50h), the majority of aggregates range in size 2−5 nm. A few aggregates ranges in size up to 20 nm. Panel C (97h) after onset, many fibrils with varies lengths ranging 100−600 nm with heights 4−7 nm. Some fibrils are stacked upon each other. Only a small amount of aggregates are present. Panel D (120h) shows a large similar shaped amount of aggregates range 2−7 nm. The protofibrils seen here are in range 450−1000 nm, with heights 2−5.5 nm. The more “mature” fibrils have lengths up to 2000 nm, heights between 9−14 nm. Panel E (144h) shows many amorphous aggregates with heights 10−20 nm. A small amount of “free” fibrils ranging in heights between 2−8 nm are also detected. Panel F (170h) shows significant changes, the mica is uniformly covered with small aggregates ranging 1−3 nm in heights. Also a few larger clusters formations are present.

Figure 4.6. Panels A-F shows tapping mode AFM images of the aggregation process for wild type Aβ(1−40) with added Sodium Chloride. All images are colour coded, brighter colour corresponds to larger heights.
4.5.1 Probability histograms of aggregate heights for Aβ(1–40)wt with the added Sodium Chloride

Figure 4.7. Histograms C-F shows the probability and particle distribution of aggregate/fibril for wild type Aβ(1–40) with added Sodium Chloride.
4.6 Aggregation process for Aβ (1−40)wt with the added Al-citrate complex

The panels displayed below shows the aggregation process over time. In all the panels the height and length of aggregate/fibril are measured. Panel A (48h) after onset, no ordered structure are present. A major group of aggregates are in the range 2–5 nm, others in range 5–15 nm. Panel B (73h) no significant changes. Panel C (98h), most aggregates are in the range 3–6 nm, the rest in the range 1–17 nm, no indications of particular structure. Panel D (120h), many protofibrils with lengths ranging 20–200 nm, also large mature fibrils with lengths up to 800 nm were detected, only a small amount unregular sized aggregates present. Panel E (144h), large mature fibrils, two types observed, one type straight and short, the other longer and with the characteristic repeating height changes. Nearly all aggregates have vanished. Panel F (48d), only one type of fibrils present, a large part of fibrils are congregated in clusters. Panel G is enlarged from Panel F.

Figure 4.8. Panels A-G show tapping mode AFM images of the aggregation process for wild type Aβ(1−40) with added Al-citrate complex. All images are colour coded, brighter colour corresponds to larger heights.
4.6.1 Probability histograms of aggregate heights for Aβ(1–40)wt with the added Al-citrate complex

![Histograms C and D showing the probability and particle distribution of aggregate/fibril for wild type Aβ(1–40) with added Al-citrate complex.](image-url)

**Figure 4.9.** Histograms C and D show the probability and particle distribution of aggregate/fibril for wild type Aβ(1–40) with added Al-citrate complex.
4.6.2 Fibril characterization for Aβ (1-40)wt with the added Al-citrate complex

The panels displayed shows together with the belonging topographic diagrams the different fibril types present with added Al-citrate. Panel A (day6), coiled fibril with maximum height 8.3 ± 0.5 nm, minimum height, i.e. the height between the twist, was found to be 4.0 ± 0.5 nm. Panel B (day6), coiled fibril, maximum height 7.2 ± 0.5 nm, due to the closeness of twists no measurements of minimum height is presented. Panel C (day48), coiled fibril, maximum height 11.9 ± 0.5 nm, minimum height 4.2 ± 0.4 nm. Distance between twists 200-275 nm.

Figure 4.10. Panels A-C with corresponding height contour diagram shows fibrils of wild type Aβ(1-40) with added Al-citrate complex. All images are colour coded, brighter colour corresponds to larger heights. The scales in the height contour diagrams are, vertically (nm) and horizontally (µm).
4.7 Aggregation process for fresh Aβ (1–40)wt

The panels below show the aggregation process over time. In all the panels the height and length of aggregate/fibril are measured. Panel A (24h), no ordered structure present, small aggregates are evenly spread across the mica surface with heights in the range 2–15 nm. Panel B (48h) a majority of aggregates ranging 3–7 nm. Panel C (72h), some larger aggregates with range up to 14 nm while a large group is in the range 3–8 nm. Panel D (98h) the majority of aggregates are in the range 3–6 nm. Panel E (120h), large fibrils present ranging between 6–9 nm, aggregates ranging between 2–5 nm.

Figure 4.11. Panels A-F shows tapping mode AFM images of the aggregation process for fresh wild type Aβ(1–40). All images are colour coded, brighter colour corresponds to larger heights.
4.7.1 Probability histograms of aggregate heights for fresh Aβ (1−40)wt

Figure 4.12. Histograms A, C, D and F shows the probability and particle distribution of aggregate/fibril for fresh wild type Aβ(1−40).
4.7.2 Fibril characterization for fresh Aβ (1−40)wt

Panel A (day 5) displayed below shows together with the belonging topographic diagram a coiled fibril with maximum height $8.9 \pm 0.7$ nm, minimum height, i.e. the height between twists, was found to be $3.3 \pm 0.9$ nm.

Figure 4.13. Panel A with corresponding height contour diagram shows the fibril characterization of fresh wild type Aβ(1−40). Image is colour coded, brighter colour corresponds to larger heights. The scales in the height contour diagrams are, vertically (nm) and horizontally (µm).
Chapter 5

Discussion and Future work

5.1 Discussion

All data presented in the preceding chapter demonstrate the effectiveness of in vitro AFM tapping mode observations. We have successfully observed the aggregation paths for synthesized wild type Aβ(1−40) in three different physiological relevant buffer solutions.

The development of an external image acquisition program was successful with respect for the increased image resolution and the user friendly GUI. The program should have been used in the early aggregation stages, where the increased image resolution up to ten times in one direction could have revealed more of the aggregates underlying structures.

The experiment with Aβ(1−40) in a standard buffer solution was a control to verify previous results by Hellberg et al. [4], due to fact that we use the same experimental equipment and setup, also of utmost importance we use the same wild type Aβ(1−40) peptide previously prepared by O. Antzutkin [22].

In the work by Hellberg et al.[4], they observed and identified a intermediate phase denoted “spherical bodies”. These spherical bodies had a continuous build up and reached a maximum in numbers (“critical concentration”) on the seventh day, with a clearly narrow height range 6.6 +/-0.9 nm. Also present the seventh day from onset was short fibrils subsequently growing into larger mature fibrils while the amount of spherical bodies rapidly diminished [4].

In our work with wild type Aβ(1−40) there are no large differences within the first 48 hours, except a more narrow size distribution compared with 24 hours. One crucial occurrence is seen the third day from onset, a build up of protofibrils. These are in the same range as the aggregates from 48 hours, from this observation we assume that the aggregates must have reached a critical concentration of folded peptide necessary for protofibril formation. These results seem to be in accordance with the
alternate aggregation pathway suggested by Morgan et al. [17]. After 146 hours there are no signs of any protofibril formations, only two larger aggregate height distributions centred around 5.5 and 10.5 nm were detected. On the seventh day fibril formations are present, also a narrowing of the size distribution, in agreement with the results by Hellberg et al. [4]. From the probability histogram F (170h) one see that the aggregate distribution centred around 10.5 nm in probability histogram E (146) has vanished indicating another critical concentration of folded peptide that apparently are the building blocks for the more mature fibrils seen in panel G. These results could indicate a stepwise build up to the fully mature fibrils, where we observed two critical size concentrations. When we compare our fully mature fibril with the results from Hellberg et al. [4], we find that our mature fibril have larger height, but the distance between pitches are more narrow. From these results we conclude that the difference in aggregation path also produces different types of fibrils.

From these observations we are lead to assume that the difference in aggregation path depends on the freezing of our prepared samples. As a control we prepared a fresh sample of wild type Aβ(1−40) with the same concentration 50 µM. The result was in agreement with our assumption, there were no visible protofibrils, only a continuous build up towards fully mature fibrils. The difference in aggregation behaviour between frozen and fresh prepared samples is something that recently was observed by M. Bibl et al. [24]. They investigated the effects of five different sample pretreatments for various peptides in cerebrospinal fluid (CSF). The peptide Aβ(1−40) experienced a 22% loss of peptide when stored 6 months at −80ºC. Our peptide samples were only stored in −70ºC for a few weeks, but surely there would have been a peptide loss according to the result by M. Bibl et al. [24]. How much the concentration of peptide diminished is uncertain, but this could in fact explain the difference in aggregation path.

Harper et al. [25], were among the first to report aggregate such as protofibrils. They performed experiments with Aβ(1−40) at a concentration of 45 µM and pH 7.4. This is very interesting compared to our result. If we assume that our samples only experienced a 10% loss of peptide, that would put us in the same neighbourhood of concentration as Harper et al. [25]. This suggests that protofibril formation for wild type Aβ(1−40) is concentration dependent. Hellberg et al. [4] performed experiments showing that peptide concentration highly effects the aggregation process. The experiments were based on concentrations of 50 µM, 12.5 µM, 4.2 µM and 2.5 µM. At concentration 50 µM, a complete fibrillization were detected, whereas at 12.5 µM there were only a few fibrils observed. With our results together with the results by Hellberg et al. [4], this suggests that for protofibrils to appear the concentration is between 12.5−50 µM.

In the experiment wild type Aβ(1−40) with added Sodium Chloride we wanted to compare the differences in aggregation path and the morphology of mature fibrils with the result from wild type Aβ(1−40), solely. In comparison, the results are very similar the first 50 hours, but after 97 hours the mica plate is completely covered with fibrils, these appear to be straight and uniform with approximately the same height range as the protofibrils in wild type Aβ(1−40), with the exception of length. Both of these fibril types have the height range that could be fitted into the fibril
model proposed by Petkova et al. [18]. After 144 hours there are surprisingly many large amorphous aggregates present accompanied with some fibril formations, and the following day only one or two large aggregates. These results could perhaps be explained in an article by Raman et al.[16], where they investigated the amyloid fibril growth of B_2-microglobulin under the influence of different Sodium Chloride concentrations and different anionic species. There result clearly indicates that fibril growth depends on the salt concentration and that anion interactions play a critical role of the stability of fibrils. The anion interactions uphold a balance between hydrophobic and electrostatic interactions, whereas high concentrations of salt seem to disrupt the balance, leading to a structural reorganization into amorphous aggregates [16]. These findings seem to correlate well with our results.

In our last experiment on wild type Aβ(1−40) with added aluminium citrate we intended to compare the aggregation process with wild type Aβ(1−40) frozen, as before. It is clear that there is a more rapid aggregation path towards fibrils, fibrils were seen two days earlier than for wild type Aβ(1−40), also there were no indications or signs of protofibrils in the early stages. How come the process toward fibril growth is accelerated with addition of aluminium citrate to buffer solution? It could be the same mechanism as with the addition of Sodium Chloride, accept two essential points, the amount and anion species released. The concentration of [Cl]^- ions 145 mM and [Al-cit]^- 150 µM, a factor of thousand, clearly an insignificant amount of aluminium citrate to make it unstable and reorganize, yet a favourably amount to accelerate the process.

When we compare the mature fibrils we find that addition of aluminium citrate produces higher fibrils. A possible and simple explanation could be found in the structural model by Petkova et al. [18]. They propose that there is a lateral association of minimal structure that generate larger fibrils. This means that if there are three minimal structures associated, the result is a fibril with height close to 12 nm. This seems to be in accordance with our measured fibril heights. Also the mature fibrils have tendencies towards cluster formations which wasn’t seen in the experiment with frozen wild type Aβ(1−40). This could indicate that the many different charged side chains pointing out from the fibrils has changed their structure, due to the attachments of aluminium complexes, thus exposing a more hydrophobic part onto the solvent exposed surface, and to minimize the exposure of hydrophobic parts cluster formations is favourable.

In another article by Petkova et al. [26], where they performed experiment with Aβ(1−40) they found two different fibril types, one in a quiescent state and one agitated. The one found in the quiescent state experience a periodic twist every 50-200 nm, with height 12 ± 1 nm. These measurements correspond well with our fibril. Due to the differences in sample preparation, concentration, pH and ionic species released in the buffer solution, this could mean that the similar results are just a coincidence, or it could mean that the buffers can differ a great deal and the predestined result is still the same. Another interesting thing with the results from Petkova et al. [26], is that the fibrils found in the quiescent state proved to be more toxic to neurons than the agitated one. This raises a question, how toxic is our fibril with addition of aluminium complex?
5.2 Conclusions

We have shown that for wild type Aβ(1−40) in a standard buffer solution the aggregation process towards fibrils involves many complex steps which are important to predict. Our results show that sample pretreatment procedures which involve freezing, results in a degradation of peptide concentration. Also shown is that for wild type Aβ(1−40) the appearance of protofibrils depends on the peptide concentration.

With the addition of Sodium Chloride in the buffer solution we have shown that there is an increased aggregation process, fibrils were seen two days earlier than for wild type Aβ(1−40). Also shown is that large amounts of [Cl] ions released in the buffer solution completely reorganize the structure into amorphous aggregates.

The addition of an aluminium citrate complex in the buffer solution also increased the aggregation process, fibrils appear within the same day as for the addition of Sodium Chloride, with the exception, the fibrils are stable and show no tendency towards structural change.

5.3 Future work

First of all, the picture noise in the external image acquisition program must be removed. Secondly, the program developed also have the ability to record the cantilever deflection signal, this means that with some additional programming together with the previously developed Pulse Force Mode [4], we have the tools for force curve imaging, which should be helpful with the understanding of molecular structures.

A continued work on the wild type Aβ(1−40) with added aluminium citrate should be highly interesting. I suggest to verify that our fibril type is the same found by Petkova et al. [26]. If so, not only test the mature fibril for toxicity, if possible test the many different aggregates forming during the whole aggregations process. Hoshi et al. [14] has shown that a spherical aggregate (amylospheroid) in fact is toxic to neurons. It could be that the early aggregates forming are highly toxic and are to be considered key elements in the development of AD.
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