Journal Article

Direct Nano-Spectroscopic Verification of the Amyloid Aggregation Pathway

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Publication Date:
2018-05-07

Permanent Link:
https://doi.org/10.3929/ethz-b-000263530

Originally published in:
Experimental Procedures

Aβ1-42 samples were prepared according to the following procedure. In the first step, a 5 mM solution of Aβ1-42 (Aβ41-42; ≥ 95 % (HPLC) purchased from Sigma Aldrich) in dimethyl sulfoxide (DMSO; ≥ 99.8 % (GC), for UV-spectroscopy, Sigma Aldrich) was prepared and stored at -20 °C. In order to initiate the fibril aggregation, the Aβ1-42 stock solution was diluted to a final concentration of 100 μM using freshly prepared 10 mM HCl in milli-Q water (pH 2.0) or phosphate buffer (pH 7.4) for comparison. Then samples were incubated at 37 °C in the darkness. After appropriate incubation time samples were measured directly in solution by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) or deposited on mica or gold for AFM, and TERS imaging. Such procedure was applied in many studies as well-established method for aggregation of Aβ41-42 leading to efficient fibril formation.[1-5]

In order to monitor the formation of particular amyloid species such as oligomers, protofibrils and fibrils and accompanying molecular changes of their secondary structure AFM and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) measurements were performed, respectively, on samples aggregating under identical conditions as described above. We were following and comparing the aggregation process with surface dependent (AFM and AFM-TERS) and surface independent (ATR-FTIR) methods, which is very important aspect of these studies due to possible influence of the gold/mica surface on the aggregation scheme.

In contrast to other analytical techniques TERS, due to its high sensitivity and spatial resolution, allows mapping of individual amyloid forms in very heterogenous samples. Therefore, we could circumvent any prior purification/fraction separation by centrifugation steps during sample preparation. This thus allows us to study more biologically relevant samples.

Based on AFM and ATR-FTIR results, presented in Fig. S1 and Fig. S2 respectively, an incubation time of 36 hours was selected for AFM-TERS measurements. After 36 hours of aggregation, samples contained amyloids at various aggregation stages including oligomers, immature granular protofibrils, and mature smooth fibrils. This allowed for a mapping various amyloid forms with the same TERS tip without sample exchange. It was very important for two reasons: i) the enhancement of electromagnetic filed, which influences spectral intensity and shape, depends on the silver nanostructure form at the tip apex, therefore a comparison of data collected with the same tip is desirable, ii) TERS probes are very delicate and their ability to enhance electromagnetic filed could often change during measurement due to silver oxidation or mechanical damage. Therefore, to reduce number of operations (such as sample exchange, landing, AFM topography collection) and reduce measurement time (oxidation) we stop the aggregation at time when all those three forms were distributed equally. For the purpose, we have also avoided sample centrifugation in order obtain significant abundance of each interesting amyloid form and to recognise them by AFM and then map using TERS.

After incubation, 10 - 20 μL of solution was deposited onto a gold coated mica substrate. Substrates were prepared by sputtering 150 nm of gold on freshly cleaved mica. After 20 mins incubation time the mica disk was rinsed with 1 mL milli-Q water to remove the excess peptide and then dried under a gentle flow compressed N₂.
Figure S1. The aggregation of Amyloid – β1-42, AFM topographies of sample deposited on mica after various incubation time at 37°C in the time-frame from 0 hours to 7 days.

ATR-FTIR of aggregating Aβ1-42

ATR-FTIR spectra were collected on an Agilent 4500a portable FTIR spectrometer equipped with a triple reflection diamond ATR sample interface. Spectra were collected from droplets (10 μL) of investigated solution (100 μM Aβ1-42 solution in 10 mM HCl or in
phosphate buffer) deposited directly on ATR diamond crystal. Data was acquired at 4 cm\(^{-1}\) resolution with 64 interferograms co-added. Each measurement was repeated three times on independently prepared sample.

ATR-FTIR spectra were processed in OPUS software. Extended ATR correction was done (number of ATR reflections 3, angle of incidence 45°, mean refraction index of the sample 1.5). Each spectrum was smoothed (9 smoothing points) and corrected for the baseline (rubber-band correction with 16 baseline points, 1 iteration) in the total range of data acquisition (900 cm\(^{-1}\) – 3600 cm\(^{-1}\)).

The amide I and amide II spectral range (1780 cm\(^{-1}\) – 1480 cm\(^{-1}\)) of ATR-FTIR spectra acquired from 100 μM Aβ\(_{1-42}\) solution in 10 mM HCl are presented in Fig. S2. Two main bands are resolved in the amide I spectral region: peak at 1650 cm\(^{-1}\) attributed to mainly unordered secondary structure such as random coil of Aβ\(_{1-42}\) and band at 1628 cm\(^{-1}\) – a hallmark of β-sheet structure. An increase of the relative content of β-sheet secondary structure in comparison unordered structure was observed with an increasing incubation time.

\[\text{Figure S2. ATR-FTIR spectra of aggregating Aβ}_{1-42}\text{ in 10 mM HCl solution (pH 2.0, upper plot) or phosphate buffer (pH 7.4 lower plot) incubated at 37 °C (incubation times 0 h, 2h, 4h, 36h, 7 days).}\]

**AFM-TERS of aggregating Aβ\(_{1-42}\)**

AFM-TERS measurements were performed using an integrated Scanning Probe Microscope (SPM) and Raman spectrometer system (NTMDT, NTEGRA, Zelenograd, Russia) in an up-right configuration. In order to perform AFM-TERS experiments, commercially available Si tips (purchased from Nanosensors) were coated with 20 nm of silver. Prior to collecting a TERS map, an AFM image of a larger area (1 μm x 1 μm to 3 μm x 3 μm) was acquired in order to choose objects appropriate for TERS mapping (globular oligomers, granular protofibrils or smooth fibrils). Then a TERS map was collected from the places of our interest. A green (532 nm) Nd:YAG laser was used for excitation. The laser power, measured at the sample level, was in the range from 0.7 mW to 1.5 mW, depending on the spectral quality desired. In principle the laser power was optimised for each tip. Spectral data was acquired using a CCD camera. Usually, square regions with a size from 240 nm to 330 nm (32 x 32, or 64 x 64 pixels) were mapped. The acquisition time at one point (single pixel) was from 15 s to 60 s depending on the desired spectral quality, therefore acquisition of one map often took several hours. Therefore due to thermal drift, mapping of larger areas was impossible. By pre-heating the instrument and overnight scanning prior to the measurements, the thermal drift was reduced from initial values to 0.21 nm/min and 0.34 nm/min in x and y directions, respectively. In total, 136 TER maps were collected from 17 samples using over 70 probes. The representative areas of interest for TERS mapping were selected based of features observed on AFM topography and phase images and their precise measurements as presented in Figures S3-S5.
Figure S3. The selection of representative area for TERS mapping: AFM topography image of aggregating Aβ(1-42) incubated at 37 °C for 36h with profiles extracted crosswise mature fibrils (profiles 1-3) and segments of protofibrils (profiles 4-5).

Figure S4. The selection of representative area for TERS mapping: AFM topography image of aggregating Aβ(1-42) incubated at 37 °C for 36h with profiles extracted crosswise mature fibrils (profiles 1-5) and along mature fibril (profile 6), the fibril periodicity is in the range from 47.5 nm to 68.4 nm.

Figure S5. The selection of representative area for TERS mapping: AFM topography image of aggregating Aβ(1-42) incubated at 37 °C for 36 h with profiles extracted crosswise mature fibrils (profiles 1-2) and along two mature fibrils (profiles 3-4), the fibril periodicity is in the range from 106 nm to 118 nm, oligomer stuck to fibril is marked by pink arrow.
All AFM topographic images were flattened by a 2nd order polynomial correction using the open source software Gwyddion for AFM (version 2.47, http://gwyddion.net/).

To reduce the amount of data collected in the TERS maps and to get a better insight into spectral changes related to the sample distribution, Principal Component Analysis (PCA) and hierarchical cluster analysis (HCA) calculations were performed. The removal of cosmic rays and the statistical calculations and correlation coefficient calculations were done using Matlab (version 8.3.0, R2014a) in combination with the PLS toolbox (http://www.eigenvector.com/).

Figure S6 presents TERS maps of the distribution of β-sheets in Aβ1-42 fibrils aggregating in phosphate buffer (pH 7.4). A disordered distribution (not linear) of β-sheets was detected in segments of protofibrils and in oligomers.

**Figure S6 Distribution of β-sheets in Aβ1-42 fibrils aggregating in phosphate buffer (pH 7.4): (A) typical spectra of turns and unstructured coils (green) and β-sheet (blue) and (B) their second derivatives, (C-D) AFM topographies of Aβ1-42 fixed on a gold substrate. In the zoomed areas, the distribution of correlation coefficient calculated from TER maps imposed on AFM topographies and derivatives of spectra (extracted from TER maps), which are the most similar to derivatives of TER marker spectra of Aβ1-42 in turns and random coils (green) and β-sheet (blue) conformation.

**Principal Component Analysis of AFM-TERS maps**

A mature fibril present in the AFM topography was mapped by TERS. The total intensity in the spectral range from 1730 cm\(^{-1}\) to 680 cm\(^{-1}\) is presented in Fig. S7b. In order to reduce the data dimensionality and end extract useful information PCA was applied. Fig. S7 c demonstrates the main results of PCA: the distribution of each score and the corresponding loading plots show the bands typical for each score. PC-1 explains 58% of total variance and is positively correlated with typical protein bands such as the phenylalanine ring breathing mode at 1004 cm\(^{-1}\), the amide III band (out of phase N-H bending coupled with C-N stretching) at 1256 cm\(^{-1}\), CH\(_3\), CH\(_2\) scissoring motions at 1428 cm\(^{-1}\), and C=O/N-H bending at 1364 cm\(^{-1}\). A small contribution from the amide I mode at 1644 cm\(^{-1}\) is also visible. A significant baseline influence is clearly visible in the map of PC-2 (explaining 5% of the total variance). The total signal is stronger in the bottom part of the PC-2 map and decreases with exposure/scanning time during mapping. The enhancement of the electromagnetic field may change/decrease during data acquisition, possibly due to an oxidation and/or due to damage of the delicate metal nanostructures on the tip as the scan proceeds.\(^\text{[9]}\) Except for visualizing the baseline influence, PC-2 also allowed resolving fibrils by the Phe and amide III bands at 1004 cm\(^{-1}\) and 1260 cm\(^{-1}\) respectively. PC-3 explains 4% of the total variance and is negatively correlated with amide III at 1256 cm\(^{-1}\) (β-sheet conformation) and positively related with the same band at 1258 cm\(^{-1}\) (turns and unstructured coils). The amide III band at 1258 cm\(^{-1}\) is distributed in the central part of the fibril and at 1250 cm\(^{-1}\) in at the fibril edges. This significant shift of the amide III is related to different conformations in the central and peripheral parts of the fibril, and probably modulated by the particular orientation on the substrate. The map of PC-4 (explaining 2% of the total variance) is positively correlated with the distribution of non-aggregated peptide mainly unstructured (amide III at 1256 cm\(^{-1}\)).
Figure S7. PCA analysis of a TERS map: (A) AFM topography of Aβ<sub>1-42</sub> fixed on gold-coated mica after 36 h of aggregation. (B) The integrated TER signal in spectral range 1730 cm<sup>-1</sup> - 1680 cm<sup>-1</sup>. (C) PCA of map presented in b: four maps of principal components (PC-1, PC-2, PC-3, PC-4 from bottom to top) together with the corresponding loading plots presented on right side of each PC.

STM-TERS of aggregating Aβ<sub>1-42</sub>

Electrochemical etching of silver wire (diam. 250 µm, ≥ 99.998%, Alfa Aesar Premion) was used to prepare tips. According to the procedure described by Stadler et al.<sup>[10],[11]</sup> each tip was immersed approx. 1 mm into the etching solution (a 4:1 (v/v) mixture of ethanol (≥99.8%, Fluka) and perchloric acid (70%, Sigma Aldrich)) in the middle of a platinum ring (diam. approx. 1 cm). This wire served as counter electrode under a potential of 10 V and a current of 10 mA. An electronic control circuit (ETHZ) cut the applied voltage as soon as the tip loses the contact with the solution. Etched tips were rinsed first with milliQ water (NANOpure DiamondTM, Barnsted) and then with ethanol.<sup>[10],[11]</sup>

STM-TERS measurements were performed using the same system as for AFM-TERS. Measurements were acquired in a constant current mode (bias voltage in the range from 0.01 to 0.02 V, with a set-point in the range from 0.004 to 0.04). For Raman excitation a focused 632.817 nm HeNe laser (red) or a 531.711 nm solid-state laser (green) were used. All spectra presented were collected using
an acquisition time between 15 s and 60 s per spectrum with the laser power of 0.3 – 1.6 mW (531.711 nm) / 0.025–1.2 mW (632.817 nm) on the sample stage. Samples prepared according to the procedure described in supplementary materials section 1 were also studied by STM-TERS. Single spectra are presented in Fig. S8. The spots where the STM tip was located during spectra collection are marked by corresponding colors on the STM topography. Poor and negative contrast clearly visible on the STM topography is related with a poor conductivity of the investigated sample. The STM-TERS signal was also weaker in comparison with the AFM-TERS spectra. The reproducible and intense peak related to the phenylalanine ring-breathing mode at 1004 cm\(^{-1}\) dominates each STM-TER spectrum. Other bands typical for proteins such as amides or C-H/N-H motions are mainly not well resolved. The high enhancement of phenyl ring vibrations in STM-TERS (gap mode) was already discussed by Blum et al. Paulite et al. applied STM-TERS for first time to map A\(_{\beta}\) by an integration of the phenylalanine ring breathing mode. The spectra presented in their work are very consistent with data acquired by us. Due to the poor enhancement of marker bands that could provide information about the protein conformation such as the amide III or the C\(_{\alpha}\)-H/N-H bending modes, STM-TERS was not used here for nano-spectroscopic mapping.

**Figure S8.**
STM-TER spectra of A\(_{\beta}\) with the points where spectra were collected marked on the corresponding STM topography.

**AFM-TERS spatial resolution**

In order to calculate the spatial resolution a knife-edge method was applied to obtained data. TER signal integrated in spectral range of Phe. ring breathing mode from 996 cm\(^{-1}\) to 1010 cm\(^{-1}\) is presented in Supplementary Fig. S9. From this TER map two intensity profiles were extracted along x (gray line, right plot Fig. S9) and y (blue line, left plot Fig. S9) axes. Sharp borders at edges of fibril are clearly visible on extracted profiles. To accurately estimate the spatial resolution, we calculated a first derivative of each section and, we fitted Gaussian functions to derivative data. The full width at half maximum (FWHM) is related to the sharpness of the TER map and it is an equivalent of the spatial resolution. Obtained values of FWHM were in the magnitude of the AFM tip apex size and equal to 15-20 nm. However, in our case, the pixel size rather than the tip size determines the spatial resolution. According to the Shannon-Nyquist theorem, the resolution that can be obtained in a raster map cannot be higher than twice the pixel size \(15\) which in our map was \(=20.6\) nm.
Fig. S9. Spatial resolution of TER map integrated in spectral range from 996 cm\(^{-1}\) to 1010 cm\(^{-1}\) with two intensity profiles extracted along x (grey line, right plots) and y (blue line, left plots) and their second derivatives; The FWHM of Gauss function fitted to the derivative of TER signal at the sharp fibril boundary is an equivalent of the spatial resolution.
References


Author Contributions

E.L., R.Z., and B.R.W conceived and designed the experiments; E.L. performed the TERS experiments; E.L. and D.P.G analysed the data; J. K. and E. L. collected AFM topography images; E.L., R.Z. and B.R.W. wrote the manuscript. All authors contributed to correcting the paper and to scientific discussions related to the data interpretation.