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Raman imaging reveals accumulation of hemoproteins in plaques from Alzheimer's diseased tissues

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KEYWORDS. Alzheimer's disease, heme, Raman imaging, Infrared imaging.

Supporting Information Placeholder

ABSTRACT: Hemes have been suggested to play a central role in Alzheimer's disease since they show high peroxidase reactivity when bound to Amyloid beta peptides, leading to the production of reactive oxygen species. Here we used Fourier transform infrared and Raman imaging on Alzheimer's diseased mice and human brain tissue. Our finding suggests the accumulation of hemes in the senile plaques of both murine and human samples. We compared the Raman signature of the plaques to the ones of various hemeoproteins and to the hemin- $A\beta$ -42 complex. The detected Raman signature of the plaques does not allow identifying the type of heme accumulating in the plaques.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder and cause of dementia. The disease is patho-physiologically characterized by aggregated amyloid-beta ($A\beta$), neurofibrillary tangles composed of aggregated hyperphosphorylated tau protein and neuronal loss. There is cumulative evidence that oxidative stress plays a key role in the pathogenesis of various neurodegenerative disorders including AD.¹⁻² For instance, the level of heme oxygenase, an enzyme that catalyzes the subsequent degradation of free heme to carbon monoxide, was found to be increased in senile plaques³⁻⁴ and an abnormal level of free hemes was suggested to increase the production of toxic Reactive Oxygen Species (ROS) and reactive nitrogen species. Since then, interest in heme- $A\beta$ complexes capable of generating toxic ROS has been growing.⁵⁻¹³ Importantly,

hemes that are overproduced inside the brain cells of AD patients⁵ have to reach the extra-cellular domain to bind $A\beta$ peptides. These free hemes (i.e. not bound to the scaffold of a hemoprotein) may be escaping apo-proteins and other heme binding biomolecules and this process will depend on the transport and delivery.¹⁴⁻¹⁵ Alternatively, free hemes can arrive to the outer side of the membrane as hemes bound to the scaffold of hemoproteins such as hemoglobin (Hb) and neuroglobin (Ngb). This is in line with the increased levels of hemoglobin (Hb)¹⁶⁻¹⁷ and neuroglobin (Ngb)¹⁸ in the brain of AD patients, found colocalized with $A\beta$ plaques. Thus, free type- α ^{5, 19} and type- β ⁵ hemes extracted from the brain of AD patients can be the result of the overexpression of hemes inside the cells or the accumulation of type- α and type- β hemoproteins. It should be noted that previously used heme extraction methods^{5, 19} allow the extraction of labile hemes bound to hemoproteins but not the covalently-attached type- γ heme^{5, 19} such as in cytochrome c (cyt c) and information on the role of these type of hemoproteins is missing. This motivated us to characterize the heme- $A\beta$ complex within the AD plaques by means of infrared and Raman microspectroscopies. We used the former to gain information about the conformation of proteins through the so-called amide I band between 1700 and 1600 cm^{-1} . This signal is sensitive to the secondary structure of proteins up to the point of differentiating parallel and antiparallel β -sheets.²⁰ The latter helps is a highly complementary technique to infrared imaging. We used it for detecting hemes through excitation at 532 nm, which is resonant with the α band of hemes.

MATERIALS AND METHODS

Behavioral characterization of AD transgenic mice and murine tissue preparation. The procedures involving animals and their care were conducted in compliance with a European Communities Council Directive (86/609/EEC) and under the supervision of authorized investigators. The protocols were reviewed and approved by the Alsace Head Office of the French Department of Veterinary and Public Health Guide for the Care and Use of Laboratory Animals. We used female APPSWE hemizygote mice [B6, SJL-Tg (APPSWE) 2576 Kha, tested for heterozygous RD1, Taconic Europe, Denmark]. These Tg2576 mice, which carry a transgene coding for the 695-amino acid isoform of human Alzheimer's beta-amyloid precursor protein, are well characterized as developing A β plaques and progressive memory deficits with age, making them suitable for the investigation of the relationship between A β accumulation/degradation and cognitive performance.²¹⁻²³ We obtained the Tg2576 mice and their wild-type counterparts at the age of 6 months, and after 4 weeks of adaptation to our local animal facilities they were submitted to monthly behavioral analyses (from month 6 to 18) using the spatial novelty task to assess their cognitive performance. This task is based on the spontaneous tendency of mice to explore preferentially those objects which have been displaced within a familiar arrangement of objects. Tg2576 mice are deeply impaired in this task, independently of the rd mutation.²⁴⁻²⁵

The mean recognition index (RI in % $\pm$ SEM: standard error of the mean) calculated according to the formula: [RI = (exploration time of the displaced object / total time exploring the displaced and non-displaced objects) * 100], during the first 5 minutes of the retention trial is shown in **Figure S1** (See **Supporting information**). At the age of 6 months, all groups detected spatial novelty. At 12 or 18 months, Tg2576 mice were deeply impaired in the task: * $p < 0.05$ compared to the wild-type mice (Student's t test).

After the behavioral characterization, 18 months old Tg2576 and wild-type mice with severe cognitive deficit were killed, decapitated, and their brains were removed and rapidly transferred to an optimal cutting temperature medium and frozen by immersion in isopentane (cooled at -40 °C). Cryo-sections (10 μ m thick) of frozen murine brains were made with a Reichert-Jung, LEICA instrument and mounted on CaF₂ or BaF₂ windows for Raman and infrared measurements, respectively. In total, 6 Tg2576 and 6 wild-type mice were included in this study.

Labeling with thioflavin S (**Figure S2**) as described by Sun, A. *et al.*²⁶ and measurement with fluorescence was performed to confirm that the examined changes in the tissue correspond to plaques (**Figure S3**).

Human tissue preparation. The human brain tissues were a kind gift from the biological resources platform of the "Hôpital de la Pitié-Salpêtrière" Paris, France. 30 μ m thick cryo-sections of the hippocampus were deposited on CaF₂ or BaF₂ windows for Raman and infrared measurements, respectively. Three hippocampi samples of AD deceased patients and one sample of a non-AD deceased patient were included in this study. The details of the human pathologies are shown in **Table S1** (**Supporting information**). Plaques were identified in the tissue first by inspection with the microscope attached to the infrared and Raman instrument respectively (see below). **Figure S4** shows the thioflavin S staining of the hippocampus of an AD patient.

Preparation of commercial samples. Cyt c from horse heart and Hb from equine heart, and hemin were purchased from Sigma-Aldrich and used without further purification. Human Ngb was purified as described in reference ²⁷. The A β 42 HCl salt was purchased from rpeptide as lyophilized powder and used without further purification. The preparation of the hemin-A β 42 complex was carried by dissolving hemin in 0.1 M NaOH and the A β 42 in a

phosphate buffer at pH 7.0. The pH of the hemin solution was brought down progressively to around pH 7 to avoid aggregation. A 1:1 ratio hemin-A β 42 solution was then prepared, and the formation of the ~1:1 ratio complex was verified by following the modification of the Soret band in UV-Vis spectral range.⁶

For Raman spectroscopy, the different hemoproteins were dissolved in water at a concentration of about 2 mM. Excess of sodium dithionite was added in diluted protein samples to obtain the reduced forms. Note that the reduction of the heme samples was necessary to compare with the tissue samples that exhibit a typical reduced heme signature, probably photo-induced by the used laser (see below).

Spectroscopy

Optical microscopy: The optical microscopy image was obtained with a Leica DMR-X microscope and a Nikon Coolpix 995 camera in phase contrast mode.

Infrared microspectroscopy: Synchrotron FTIR microspectroscopy was performed at the beamline IR2 of the ANKA synchrotron facility (KIT, Karlsruhe, Germany) on an IRscope II infrared microscope (Bruker Optics, Germany). Infrared spectra were recorded using a 36x objective in transmission mode in the 700-4500 cm⁻¹ spectral range with a spectral resolution of 4 cm⁻¹, 128 co-added scans, 12.5 μ m aperture dimension and a step size of 4.16 μ m. A background spectrum was collected from a clean BaF₂ window after every 10 spectra. FTIR spectra and maps were analyzed using Cytospec v 2.00.01.

Raman spectroscopy: Raman spectroscopy was performed on a WITec Alpha300RS confocal Raman microscope using a 532 nm laser with a resolution of 2-3 cm⁻¹ and with 600 lines/mm. Raman spectra of mice tissue samples were recorded using a 20x/NA 0.4 objective in the 0-3800 cm⁻¹ spectral range with an integration time of 0.05 s. The human samples were studied with the same microscope using a 50x/NA 0.75 objective in the 0-3800 cm⁻¹ spectral range with an integration time of 1 s. A spectrum was recorded every 0.5 μ m along the x and y directions. Raman spectra of model samples were recorded using a 50x/NA 0.75 objective in the 0-3800 cm⁻¹ spectral range with an integration time of 10 s using 532 nm (WITec Alpha300RS Raman microscope). The baseline of the Raman spectra of the isolated cyt c and Ngb were corrected due to the high fluorescence. The resolution of the later instrument was 1200 lines/mm, corresponding to about 1 cm⁻¹. Raman spectra and maps were analyzed using WITec or the Renishaw software, respectively.

RESULTS AND DISCUSSIONS

Infrared microspectroscopy. The amide I and amide II signals measured for diseased and normal mice and human brain tissues are shown in **Figure 1A** and **1C**, respectively. These amide signals correspond to the C=O and the coupled CN/NH vibrational modes of the protein backbone. The position of the C=O vibrational mode corresponds to a specific secondary structure.²⁰ A transition of the amide I peak from 1662 cm⁻¹ (black line, normal mice tissue) to 1630 cm⁻¹ (red line, diseased tissue) is seen. The shift corresponds to the expected accumulation of peptides and proteins organized in parallel β -sheet type structures.²⁸ In AD, this accumulation is related to the aggregation of A β .²⁹ The map obtained by infrared microscopy (**Figure 1B**) shows the intensity distribution of the 1630 cm⁻¹ signal, revealing the strong concentration of parallel β -sheet type structures in the core of the plaque (bright color in the map) and its absence in the surroundings (dark color). Moreover, the downshift of the amide II from 1550 to 1542 cm⁻¹ can be associated with the increase β -sheet content,³⁰ i.e. A β aggregates. The signal at 1747 cm⁻¹ observed in panel A can be assigned to the

ester C=O stretching vibration of phospholipids.^{31–33} In line with previous studies, the absence of the phospholipids signals in the plaque core suggests that phospholipids form a ring around the aggregated A β .^{34–36}

It should be noted that the plaques were identified by staining with thioflavin S (**Figure S2**). The microscopic image of a typical sample of the hippocampus from 18 months old Tg2576 mice is shown in **Figure S3**. All aggregates studied here have been found to be clearly distinguishable from the normal tissue and to show highly reproducible spectral properties (**Figure S5**). Their size is typically 10 μ m for 18 months diseased mice.

In analogy to the mice sample, a human AD sample was examined by infrared microspectroscopy. **Figure 1C** shows the amide I and II modes obtained from human AD brain tissue. The spectrum (black trace) recorded away from the senile plaque shows a maximum at 1655 cm^{-1} and a shoulder appears at 1634 cm^{-1} in the spectrum of the plaques (red trace). This shoulder can be assigned to the accumulation of aggregated A β . Clearly the spectra of healthy and diseased parts of the human sample differ from the murine one especially regarding the sharp signal appearing in the murine sample at 1630 cm^{-1} whereas the human sample shows a shoulder at 1634 cm^{-1} . The observed difference between the murine and human samples is most likely caused by the difference in composition of tissue between humans and mice^{37–38} as well as by the thickness of the sample; the human sample is 3 times thicker than the murine one, hence it is more difficult to observe the markers of the senile plaques against a thicker healthy tissue in the background. **Figure 1D** shows the infrared map of the human plaque obtained at 1634 cm^{-1} . A higher intensity at 1634 cm^{-1} is found in the core of the plaque, in line with the data obtained for the murine sample.

Figure 1. Infrared spectra of murine (Panel A) and human (Panel C) brain tissue away from the plaque (black line) and on the plaque tissue (red line) in the 1800–1500 cm^{-1} spectral range. The infrared maps of the murine (Panel B) and human (Panel D) obtained on the basis of the intensity distribution of the specific band (circled wavenumber). Bright color corresponds to a high intensity and dark color to a low intensity. The FTIR spectra were obtained at ANKA IR beamline (For details see **materials and methods**).

Raman microspectroscopy

Figure 2A shows the Raman spectra obtained in the spectral range 1800 to 600 cm^{-1} for the murine senile plaque (red) in comparison to the healthy tissue (black). Several sharp and strongly enhanced signals can be identified in the spectrum. For instance, the signals at 1587, 1366, 1132 and 754 cm^{-1} are all assigned to hemes modes.³⁹ **Figure 1C** displays the relative intensity distribution of the signal observed at 1587 cm^{-1} (assigned to the antisymmetric $\nu(\text{C}=\text{C})$ vibration of the heme skeleton (i.e. ν_2)).⁴⁰ The intensity of this signal is about 3.3 ± 1.3 higher in the plaque relative to the healthy tissue as assessed from eight different samples. Noticeably, a quite homogeneous distribution of the contribution is found in the plaque, with a high intensity found in the entire plaque and low signal intensity in the surrounding tissue. **Figure 2D** shows the analysis of three different samples from patients who suffered from AD as well as a control sample taken from non-AD person (See **Table S1**). Several of the heme marker bands identified in the murine samples can be found also in the human tissue. It is noted that the signals are highly similar between the tissues of the different patients, confirming their accumulation in the plaque of AD patients. The mapping of the tissue at 1590 cm^{-1} reveals the distribution of hemoproteins in the entire plaque.

Figure 2. Raman mapping of the murine and human samples. Panel A shows the average spectra of the murine plaques (red trace) and its surrounding (black trace). Panels B and C show respectively the corresponding white light and Raman image at 1587 cm^{-1} . Panel D shows the average spectra of human plaques. Traces 1–3 correspond to three human AD samples and trace 4 corresponds to a non-AD control as described in **Table S1** of the **Supporting information**. Panels E and F show respectively the corresponding white light and Raman image at 1590 cm^{-1} . The Raman images were obtained with an excitation at 532 nm; NA 0.4; 11 mW at 2–3 cm^{-1} resolution. The spectra are off-set for clarity. The bright color corresponds to a high intensity and dark color to a low intensity.

The Raman data in **Figure 3** show the direct comparison of the tissues (human and murine) with reduced Hb, reduced Ngb, reduced cyt *c*, as well as a hemin-A β 42 complex. Resonance Raman spectroscopy is specific for the exact type of ligation, spin and redox state as well as the environment of the porphyrin ring and can thus be used to identify and distinguish the heme type.^{39, 41} The signal found at 1366 cm^{-1} in the spectrum of the murine plaque and at 1362 cm^{-1} in the spectrum of the human plaque is a marker

of reduced heme (or hemoprotein).³⁹ The characteristic signals that may be arising from the hemoproteins (or free hemes) in the tissue are traced with vertical lines. Several signals detected from murine and human plaques may be correlated with modes of a low-spin 6-coordinated heme.⁴¹ In particular the ν_2 mode at 1587 cm^{-1} is a marker for the spin state of the heme where the hemoprotein is in the low-spin state. The ν_3 mode, observed at 1501 cm^{-1} in the spectrum of the murine sample and at 1505 cm^{-1} in the spectrum of the human sample indicate that the heme is hexa-coordinated. The wavenumber of the heme marker bands found in the various samples are shown in **Table 1**. Clearly, type-*b* (free heme, Hb and Ngb) and type-*c* hemes can be responsible for the enhanced signals in the plaques since their wavenumbers match the ones found in the tissue samples. heme *a* absorbs between 603 and 611 nm⁴² it is thus not resonant with the laser (i.e. 532 nm) used here. Therefore, even if heme *a* is present in the brain tissue samples, its contribution can be overshadowed by the contribution of the resonant type-*b* and type-*c* hemes. Thus, type-*a* heme is not discussed.

Figure 3. Comparison of Raman spectra of murine plaque (green line), human plaque (black line), hemin-amyloid beta 42 complex (grey line), reduced neuroglobin (blue line), reduced hemoglobin (red line) and reduced cytochrome *c* (magenta line). The spectra are obtained with excitation at 532 nm, NA 0.4: 11 mW, at 2-3 cm^{-1} resolution. The vertical lines highlighted the frequency of characteristic bands from the murine plaques to facilitate the comparison to the other spectra.

Table 1. Raman shift in cm^{-1} of the heme modes in the different samples.

Assignmen t_{39-41}	ν_2	ν_3	ν_4	ν_{14}	ν_{15}
Brain tissue					
Mice	1587	1501	1366	1132	754
Human	1590	1505	1362	1130	752
Type <i>b</i> heme					
Hemin +A β 42	1584	1501	1370	1128	749
Ngb	1587	1500	1369	1132	749
Hb	1584	1496	1356	1128	748

Type <i>c</i> heme					
Cyt <i>c</i>	1586	1500	1366	1131	751

In line with previous results, Hb¹⁶⁻¹⁷ can be responsible for the observed Raman marker bands. The potential accumulation of cyt *c* in the senile plaques could result from the release of cyt *c* due to apoptosis⁴³ and could act as a signaling molecule for brain microglia leading to regulate the immune response.⁴⁴ Ngb was found upregulated in AD brains and previously detected accumulating in plaques.¹⁸ Some in vitro studies and cell culture experiments suggest that neuroglobin may detoxify ROS or nitric oxide and when accumulated in the tissue it may be present as a reaction to the cell's death and the increased ROS produced. Interestingly Ngb was suggested to protect neurons from A β -induced neurotoxicity as Ngb overexpressing transgenic mice were resistant to the toxic effects.⁴⁵ Thus, it would not be surprising to find Ngb in the senile plaques. Interestingly cyt *c* is a binding partner for neuroglobin⁴⁶⁻⁴⁷ and it was shown that a fast electron transfer between the heme iron centers in ferrous Ngb and ferric cyt *c* takes place, proposing a fast association of Ngb to pro-apoptotic cyt *c*.⁴⁸⁻⁴⁹

Consequently, important questions in AD research field rise: i) why do hemes and/or hemoproteins accumulate in A β plaques and not in the surrounding tissue? Is this a result of the aggregation cascade? ii) does this accumulation have a beneficial role by enhancing the brain immune system to clear the plaques? or iii) does it have a damaging role in accelerating the plaques formation by increasing the production of ROS?

CONCLUSIONS

In this study we investigated the vibrational signature of AD plaques from mice and human samples. FTIR imaging highlighted the presence of β -sheet aggregates of A β peptides in the senile plaques of both murine and human samples. Raman imaging at 532 nm confirms the accumulation of heme in the plaques but the similarity of Raman signature of free hemin bound to A β -42 and type-*b* and type-*c* hemoproteins does not allow ruling out one or the other hemoprotein. Therefore, free hemes or Hb, Ngb and cyt *c* hemoproteins could be accumulating in the A β plaques. Due to the suggested implication of the heme in AD pathophysiology, we believe that it is necessary to further investigate the type of the heme to provide solid proof on the accumulation of either type of hemoproteins in AD plaques and to shed more light on the binding mode of A β to the heme moieties.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Mice behavioral analysis, the Braak stage of the human subjects, plaque thioflavin *S* staining and additional Raman imaging data of mice plaques (PDF).

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Author Contributions

Youssef El Khoury and Alicia Schirer performed the spectroscopy experiments, prepared the figures and prepared the manuscript, Christine Patte-Mensah, Christian Klein, Laurence Meyer and Monika Rataj-Baniowska have been in charge of the mice and the preparation of the tissues, Sophie Bernad, prepared the Neuroglobin samples, David Moss, supervised the IR experiments at the synchrotron in Karlsruhe and supported data analysis, Sophie Lecomte, supervised the Raman imaging and supported the data analysis, Ayikoe-Guy Mensah-Nyagan, supervised the mice experiments and supported data analysis and Petra Hellwig, coordinated the collaboration and supported the preparation of the manuscript. All senior authors raised funding All authors reviewed the manuscript.

Note

The authors declare no competing financial interests.

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ABBREVIATIONS

Aβ, amyloid beta; AD, Alzheimer's disease; Cyt c, Cytochrome c; FTIR, Fourier transform infrared; Hb, Hemoglobin; Ngb, Neuroglobin; ROS, Reactive Oxygen Species;

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