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Confined laminar flow on a super-hydrophobic surface drives the initial stages of tau protein aggregation

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Abstract

Super-hydrophobic micro-patterned surfaces are ideal substrates for the controlled self-assembly and substrate-free characterization of biological molecules. In this device, the tailored surface supports a micro-volume drop containing the molecules of interest. While the quasi-spherical drop is evaporating under controlled conditions, its de-wetting direction is guided by the pillared microstructure on top of the device, leading to the formation of threads between the neighboring pillars. This effect has been exploited here to elucidate the mechanism triggering the formation of amyloid fibers and oligomers in tau related neurodegenerative diseases. By using Raman spectroscopy, we demonstrate that the fiber bridging the pillars contains β-sheets, a characteristic feature of amyloid aggregation. We propose that the combination of laminar flow, shear stress and molecular crowding taking place while the drop is evaporating on the SHMS, induces the reorganization of the tau protein secondary structure and we suggest that this effect could in fact closely mimic the actual mechanism occurring in the human brain environment. Such a straightforward technique opens up new possibilities in the field of self-assembly of biomolecules and their characterization by different methods (SEM, AFM, Raman spectroscopy, TEM), in a single device.

Introduction

Tau protein is an intrinsically disordered protein (IDP) present in the human brain with its six isoforms all encoded by the Microtubule-Associated Protein Tau (MAPT) gene. Its main function is to stabilize the microtubules in the axons [1]. It was found that samples derived from patients with Alzheimer disease and other neurodegenerative diseases comprise neurofibrillary tangles (NFT), formed by paired helical filaments (PHF) of amyloid nature containing all six tau protein isoforms, but mainly the ones bearing a longer sequence [2,3]. The pathological role of tau in the onset of cognitive impairment is not yet clear though. Often, the non-soluble PHFs themselves, are considered toxic [4], whereas a recent study seems to prove that only the soluble oligomeric tau (tau dimers/trimers assembled as β-sheets) has a

degenerative effect, being able to induce amyloid-like aggregation of soluble tau monomers [5]. This study and most of the references therein seem to agree on the toxicity of the phosphorylated tau isoforms. Either way, the mechanism leading to both oligomers and tangles formation needs to be fully understood in order to rationally plan the medical treatment of the disease. We hypothesize that the laminar flow and shear stress in crowded environment are able to organize the intrinsically disordered protein tau into at least oligomers characterized by a β-sheet secondary structure component, main feature of any amyloid protein. The effect of laminar flow on the organization of functional amyloids is well represented in Nature, the mechanism of production of the spider silk being an example [6] and has already been reproduced by inducing aggregation e. g. in a fluidic chamber [7]. It has been reported that the shear flow in the interstitial fluid and blood vessels of the brain could have a role in the amyloid-β aggregation leading to senile plaques [8] and the mechanism has been first noted and then reproduced in a simple chromatography column mimicking the blood vessels [9]. It is plausible that the same mechanism could lead to the generation of toxic tau species, given that the ability of tau to enter the brain interstitial space and to act as a prion protein has been demonstrated [10]. Other agents, together with the shear stress and the crowded environment, are supposed to play a role in the misfolding and mis-aggregation of protein species, such as, e. g., their net charge in solution and their interaction with molecules present on the cell surfaces like heparan sulfate, but were not taken into consideration in this preliminary study [11,12].

In essence, our hypothesis is that a solution with an excess of phosphorilated tau protein flowing in a liquid capillary, will experience a shear stress able to partially expose the hydrophobic core of the protein, while the laminar flow itself will guide the ordered aggregation of the destabilized protein into a new, more stable configuration such as the β-sheets. We were able to generate both the effect of a crowded environment and of the shear stress present in a viscous fluid flowing in a capillary in a single device. Similarly to previous work from our group [13,14], we here exploit the effect of a tailored super-hydrophobic micro-patterned surface (SHMS) on the controlled evaporation of a drop containing the tau monomers. A set of cylindrical pillars is arranged in circular concentric arrays spaced according to the experimental needs. Upon dropping a suitable volume of the solution on top of the device substrate, a quasi-spherical drop will form and isomeric evaporation according to the temperature and relative humidity of the surrounding environment will take place. The top surface of the pillars is the actual contact area of the drop with the substrate. Upon evaporation, the drop will maintain a quasi-spherical shape while a receding meniscus will inevitably form at the pillars top edge, and a viscous liquid capillary will be shaped in the short transient while the drop hops from one pillar to the adjacent one. This mechanism can lead to the formation of biological threads bridging the pillars whenever the right concentration of the solute in the evaporating sphere is reached. In this study in particular, we were able to produce threads of the full length isoform of phosphorylated tau protein suspended across the pillars

and we verified by Raman spectroscopy that the characteristic signature of the β-sheet secondary structure was present in the Amide I band of their Raman spectrum, suggesting the presence of amyloid-like supramolecular assemblies. We propose that the shear flow in the liquid capillary generated when the drop hops from one pillar to the neighboring one plays a crucial role in this fast re-organization of the molecules. In fact, since the solution itself did not contain amyloid fibers, not even after its evaporation, we conclude that the confined laminar flow in the tube bridging the pillars is in fact responsible for the organization of the tau protein under study, and that this mechanism could likely mimic the process leading to tau aggregation in the extra-cellular environment of the brain.

Materials and Methods

1. Super-hydrophobic Micro-fabricated device

The super-hydrophobic substrates were fabricated as previously reported [13]. Briefly, a combination of optical lithography and Deep Reactive Ion Etching (DRIE) was used to pattern the surface in frames of 3x3 mm with a circular pattern of pillars, each having a diameter of about 6 μm, while the radial pitch is 18 μm. The original substrate was a standard Si ⟨100⟩ 4" wafer. In the first step, the pillars pattern was defined by means of optical lithography, using a 2 μm thick layer of AZ5214 photoresist in negative tone. The sample was then etched in a DRIE system (PlasmaLab System 100, Oxford Instr.), obtaining a final height of the pillars of about 10 μm. Finally, the deposition of Perfluorodecyltrichlorosilane (FDTS) in a Molecular Vapor Deposition System (MVD100E, Applied MST) was accomplished to render the Silicon substrate hydrophobic. Once deposited on the substrate, the 5 μl of liquid solution create a truncated sphere of approximately 3 mm in diameter, with a contact angle of ~150^{o} and hence with a diameter of the footprint on the sample of ~1.5 mm.

2. Protein tau and thread generation

Phosphorylated tau-441 was provided by SignalChem Pharmaceuticals Inc., Canada. Uniprot (www.uniprot.org) accession number is P10636-8. The recombinant protein was stored at [0.2 μg/μl] in 50 mM Tris-HCl, pH 7.5, 150mM NaCl, 0.25 mM DTT, 0.1 mM PMSF, 25% glycerol at -80 ℃. The unwanted aggregates were spun down at 20,000 *g* for 10 minutes before usage. The surnatant was recovered and 5 μl of the solution were deposited at the center of the SHMS by using a hypodermic syringe in a humidity chamber equipped with a home-built device to control the temperature gradient. The purpose of the setup is to carefully control the evaporation of the drop. The sample with the drop was placed on a 500 micrometer thick silicon substrate whose temperature is controlled by a heating/cooling Peltier element to continuously lock its temperature at a chosen temperature difference with respect to a reference plate placed 5 cm above the sample. This was implemented in order to create a constant

temperature difference between the liquid and the external environment, thus keeping stable and uniform the evaporation rate at the surface of the drop and to establish a regular convection flux of the liquid, which favors the redistribution and rearrangement of the molecular components dissolved in solution. The entire setup was placed into a humidity controlled chamber (Mini Humidity Chamber, Cole-Parmer). The relative humidity (RH) in the chamber was kept at 97%. The difference of temperatures was set at 5 ℃ for the first 5 hours and then was increased at 10 ℃ for 17 hours. Afterwards the RH in the chamber was lowered to 50% for 5 hours. The last two steps were introduced to speed-up the final evaporation process leading to stable inter-pillar threads. Finally each sample was recovered and analyzed by either SEM, AFM or Raman spectroscopy.

3. SEM and AFM characterization of the sample

We wanted to verify if amyloid-like structure of tau protein 441-P were already formed in the residue of the tau monomers solution drop after its evaporation on the SHMS. After the incubation time required for thread generation, the drop was collected from the SHMS by a pipette, dissolved into a volume of 20 µl in MilliQ water and finally deposited over a freshly cleaved mica substrate and let adsorb for 30 minutes. The substrate was then gently rinsed three times with MilliQ water, N_2 dried and measured in the AFM at RH below 15%. AFM topography of the sample was performed by JPK Nanowizard III mounted on inverted Olympus IX73 microscope. XSC11 AFM probe (MikroMasch, Nanoworld AG) with nominal resonance frequency of about 150 kHz and nominal force constant of 7 N/m was run in tapping mode for the topography measurement.

After the drop containing the tau monomers was fully evaporated on the SHMS, the full sample was sputter coated with a 2 nm layer of Iridium in a sputter coater (QI150T, Quorum Technologies). Images were acquired by a SEM (Magellan, FEI) at an acceleration voltage of 3kV and 50 pA current.

4. Raman spectroscopy

Raman spectroscopy was performed in confocal back scattering geometry by exciting the sample with 532 nm linearly polarized laser light (Coherent Compass Sapphire Laser, 75 mW) at 2 mW power on a WiTec Raman spectrometer (Alpha300 RA) coupled with Andor CCD detector (DU970N) cooled at −65 ℃ and a 100× objective (Zeiss, EC EPIPLAN NEOFLUAR, 0.9 NA). The laser spot was centered in the middle of the dried thread or on the collapsed drop to perform the measurement. For each measurement 3 spectra were acquired accumulating 10 spectra at 30 seconds integration time each. The spectra were then baseline corrected in the range 1520-1750 cm^{-1} using a linear baseline, normalized and averaged. Amide I spectral region was fitted to a superposition of three peak functions, centered at 1655, 1671, 1690 cm^{-1} , each function being a convolution of Lorentzian and Gaussian functions based on [15].

Raman spectroscopy measurements were performed on the larger features, since the localization of the smaller features is limited by the resolution of the optical microscope.

Results and discussion

Of the six isoforms of tau protein expressed in the brain, we focused in this study on the tau-F form, 441 aminoacids in length, in its phosphorylated state (from now on tau 441-P), since it has been shown to be abundant in patients' NFTs [16]. The protein is found as a monomer in solution and it is an intrinsically disordered protein, meaning that its average secondary structure is disordered in nature, nonetheless the isoforms are found in different conformations that give rise accordingly to different functional states [17]. The mechanism leading to the aggregation of tau protein into amyloid species is currently not known: we propose that the concomitant laminar flow, shear stress and molecular crowding that could be generated in the brain environment have a role in the fibrillation process. We in fact suggest that certain areas of the brain, like the interstitial space between the cells and the vascular system, are actually a suitable place where the tau protein could be subject to aggregation, misfolding and mis-aggregation, given the simultaneous presence of crowded environment, destabilizing molecules such as e. g. proteoglycans emerging from the cell surface, and laminar flow confined in a small volume [18,19].

1. Generation of liquid capillary

To mimic the laminar flow of a concentrated solution in a micrometric environment we used a SHMS device. The principle behind the performance of the device is the following: a drop deposited on top of the SHMS device will evaporate maintaining its quasi spherical shape. Due to evaporation of the solvent, the concentration of the molecules inside the drop gradually increases. At the same time, because of the drop volume shrinking associated to the loss of water and the super-hydrophobic properties of the surface, there is a recession of the contact line on the substrate during evaporation. Due to the presence of a discretized structure of the surface, this movement of the contact line must proceed in steps. First a pillar in the vicinity of the contact line is completely covered by the solution. As the contact line tends to move beyond, the liquid tends to adhere to the pillar top for a longer time after the spherical profile moved away. The result is the creation of a liquid bridge of tubular shape between the spherical profile and the pillar top. In case the liquid solution contains some suspended molecules, like the tau proteins in our case, they might form a structure which dries after the drop regression leaving a bridge between adjacent pillars. This cannot happen if the solution is not viscous enough, meaning that the concentration of the molecules inside the drop is a discriminating factor for the generation of the bridging thread. In Fig. 1 we depict the entire process, from drop casting to final formation of the threads. In step (a), a drop of

the solution containing the tau protein monomers is deposited on top of the SHMS tailored surface where the centripetal evaporation (indicated by the red arrows) is taking place at controlled humidity and temperature parameters; in step (b), at suitable tau protein concentration several bridging structures develop following the regression of the contact line; in step (c), the final static state is reached where stable threads are formed and the drop eventually collapses in a solid residue. We are interested in better defining the step leading to the situation depicted in Fig. 1b, because it is involved in the initial stages of tau protein aggregation. Dissecting the case we can identify three phases (I, II, III) as exemplified in Fig. 1d (top view). The solution drop surface initially occupies an area defined by the pillar top (I), afterwards, while the drop is evaporating, a liquid capillary is generated from one pillar to the next, which restrains the solution into a tube having a diameter smaller than the pillars top (6 μm diameter) (II), where the laminar flow is strong due to liquid regression towards the drop and the shear stress increases at the liquid-gas interface (III). We were able to capture these events by using a camera (SI). It was possible to observe the drop meniscus sliding on top of the pillars while the drop is in its final stage of evaporation. Several threads are formed across some of the neighboring pillars in the transient when the solution meniscus hops from one pillar to the next. Although it is not possible to clearly observe the formation of the liquid capillary due to the spatial and temporal resolution limits of our optical detection apparatus, the images acquired in the SEM microscope after the drop complete evaporation reveal that the thread has a funnel shape in most of the cases. The same shape was also observed in another our previous work [13]. To exemplify the final effect of the evaporation of the liquid droplet, we report in the inset in Fig. 1d an SEM picture. It is reasonable to speculate that the funnel shape of the residuals on the pillar top is actually the shape of the original liquid tubular funnel which originated from the evaporation of the drop, however the data collected here do not allow to draw any clear conclusion on this point, which will be further detailed elsewhere.

In several works we have demonstrated that it is relatively straightforward to bridge a suitably long molecule (with length comparable to or larger than the pillars gap) across the pillars, like DNA and lysozyme amyloid fibers [13,14,20–23]. However it is not obvious to generate a thread (especially of sub-100 nm diameter) when there is no pre-formed structure in solution whose ends could anchor at the pillars edge. To further demonstrate this, we tested as negative controls, drops made of MilliQ water, a solution containing glycerol, and a buffered solution containing the globular protein hen egg white lysozyme (HEWL): the results indicate the absence of any structure hanging across the pillars after their evaporation on the SHMS. Therefore we suggest that the shear stress and laminar flow specifically acting on intrinsically disordered tau proteins have a leading effect on the initiation of the molecular self-assembly. In particular, we were driven to this conclusion by the fact that the solution containing the HEWL was not forming any thread bridging the pillars, thus suggesting that its secondary structure is not

loosen enough by the flow induced in the device. On the contrary, the disordered structure (even partial), which is an essential preliminary step before any type of amyloid-like aggregation can occur, appears to be preserved in the solution containing the tau 441-P monomers, therefore we argue that the laminar flow and induced shear stress are actually playing a role in the dynamics of the assembly.

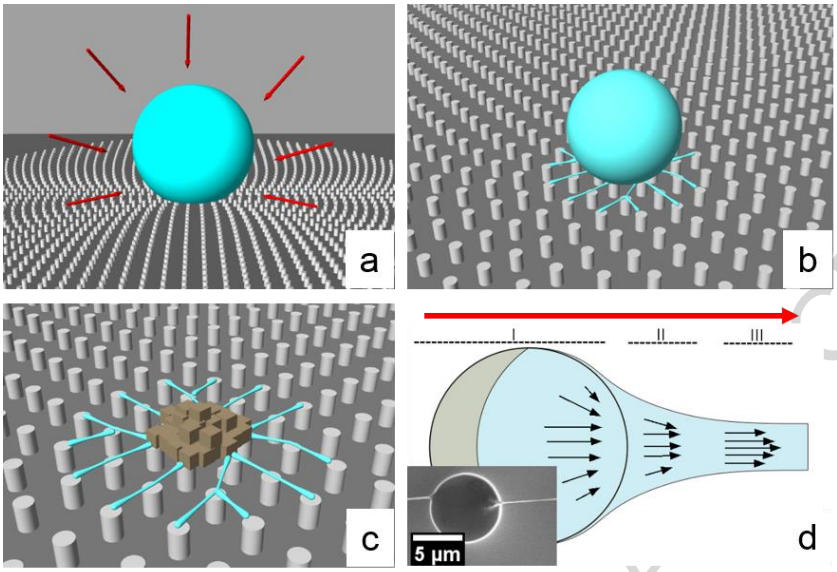

Fig. 1: Schematic representation of the tau protein threads generation on SHMS: (a) initial stage where the drop containing the tau protein is deposited on the SHMS and the centripetal evaporation proceeds in the direction indicated by the red arrows; (b) intermediate step where the drop is gradually shrinking maintaining its quasi-spherical shape and mediates the formation of the threads bridging the pillars top: they appear like liquid tubes or capillaries with a funnel shape starting from the pinch-off pillar; (c) the drop eventually collapses in the final residue, where salt crystals are visible, while the threads gain their final stable structure. In panel (d) the sketch depicts the transient in the pillars top de-wetting process, when the laminar flow is generated, related to the drawing in panel (b). The contact point moves from left to right while the drop evaporates as indicated by the red arrow. (I) the pillar top surface is occupied by the solution; (II) the solution is dragged away from the pillars top to the next one when the evaporation induced volume decrease pulls the drop away from the pillar, creating a transient liquid bridge between the pillar top, where the liquids tends to remain attached for a short time before receding, and the rest of the drop; (III) the flow inside the nanotube is fully laminar, with an increased shear stress at the air/water interface. The black arrows indicate the fluid flow direction. The inset shows and SEM image with the final evaporated thread where a funnel shape of the dry residue formed at the pillar top edge is clearly visible.

In Fig. 2, SEM images of representative threads of tau 441-P, obtained over a SHMS are presented. The gap between two neighboring pillars is 12 µm, so that we obtained threads of similar length while diameters were quite variable, ranging from ~500 nm to ~30 nm. The final collapsed drop is visible in the bottom left corner in Fig. 2a and residues of salts and amorphous material are evident on the

pillars top. In this case the threads are about 200 nm in diameter. In Fig. 2b, one of the smallest diameter threads with less than 50 nm diameter is shown. The analysis of the EM figures, suggests that the thinner threads obtained are straight, with no agglomerates, impurities or salt crystals, thus the device generates threads of almost completely pure biomolecules. To be noted, even in the smaller threads the funnel shape is evident.

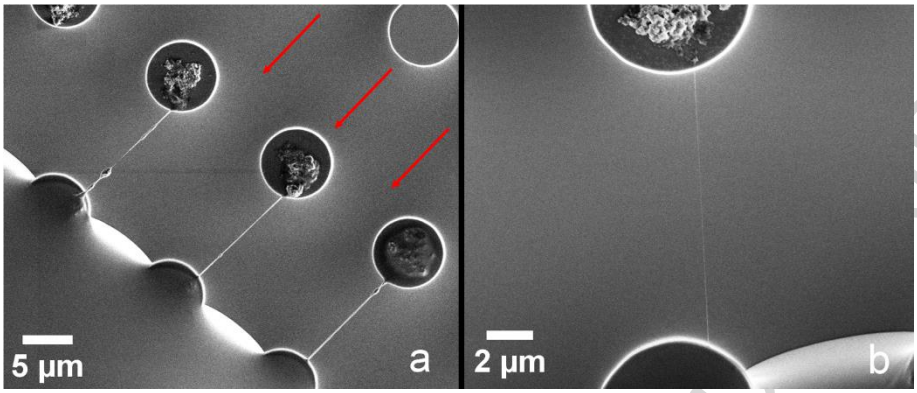

Fig. 2: (a) SEM micrograph of the SHMS with tau 441-P after complete evaporation. The de-wetting boundary where the drop finally collapsed is visible at the bottom left corner. A few threads are hanging at pillars edges (diameters in the 100-300 nm range). The red arrows indicate the evaporation direction. (b) SEM micrograph taken in another area of the same sample showing a thinner bundle (diameter <50 nm) bridging two pillars.

2. Raman spectroscopy on protein tau threads

To characterize the protein threads we chose the Raman spectroscopy technique, which is a non-destructive, label-free method, often employed to describe biological material and especially suitable to depict the secondary structure of proteins. Previously, we successfully employed the technique to characterize lysozyme amyloid fibrils stretched between adjacent pillars, clearly displaying the characteristic peak of the β-sheet secondary structure centered at ~1672 cm^{-1}, a main feature of the amyloid fibers [13]. In this work, the Amide I band in the broad range from 1570-1720 cm^{-1} was chosen to identify the secondary structure of the tau protein [24]. In Fig. 3 an example of Raman spectra measured on the thread ("thread") and on a collapsed drop area ("bulk") is plotted in the range 1520-1750 cm^{-1}. In the inset, the bright spot is the laser illuminating the thread (top) or the bulk residue (bottom). Raman measurements were performed for comparison in an area of the final drop residue that we assume is not having any ordered amyloid type of structure. The spectrum obtained on the thread is in fact quite different as compared to the one obtained on the collapsed drop. At a first glance the Amide I band appears to be up-shifted in the case of the spectrum acquired on the thread. To detail the composition in

secondary structures of the Amide I band, we performed a three band fitting with maxima centered at 1665, 1671 and 1690 cm^{-1}, which according to the literature could correspond to α-helix, β-sheet and disordered structures (PPII type) vibrational modes respectively [15,25]. We also incorporated in the fit the peaks related to the aromatic residues including the indole ring modes of Tryptophan (1556 cm^{-1}) and the ring modes of Tyrosine, Tryptophan and Phenylalanine (1620 cm^{-1}). As can be seen in the plot in Fig. 3, the band peaked at 1671 cm^{-1}, highlighted in green, is significantly represented in the spectrum acquired on the thread whereas in the case of the collapsed drop area, the fitting at 1671 cm^{-1} results in a band whose amplitude is negligible within the limits of the experimental uncertainty. We thus show that a change in the secondary structure of the tau 441-P protein occurs after it is stretched and ordered by a confined laminar flow between two pillars. To check for the presence of amyloid fibers in the bulk droplet solution, we prepared an identical sample, recovered the drop from its surface before it was fully evaporated and we measured its content by AFM topography (data not shown). We did not find any amyloid fiber of appreciable length (within the instrumental resolution limit), as compared for example to our previous work on amyloid lysozyme fibers [13]. In that case we first generated the fibers, verified their presence by AFM and deposited them on the SHMS: we showed that the fibers extended well across the pillars. The bridging of two neighboring pillars by the amyloid fibers thread was possible because the length of the fibers was exceeding the gap between the pillars: hence the de-wetting of the evaporating drop was actually "combing" the supramolecular assemblies from one edge to the next of the SHMS pillars. In this work instead we could not detect any pre-formed fiber in the solution, so that we didn't expect to have any thread containing fully formed amyloid fibers. However, because the signature of the amyloid secondary structure is indeed detected in the Raman spectra acquired on the thread, we strongly believe that the laminar flow that exists in that de-wetting region is able to organize the tau molecules, driving the conversion from their native structure to a β-sheet aggregate. This mechanism could in fact mimic an initial stage of the tau monomers aggregation, before they eventually organize into the more complex structure of the amyloid fiber.

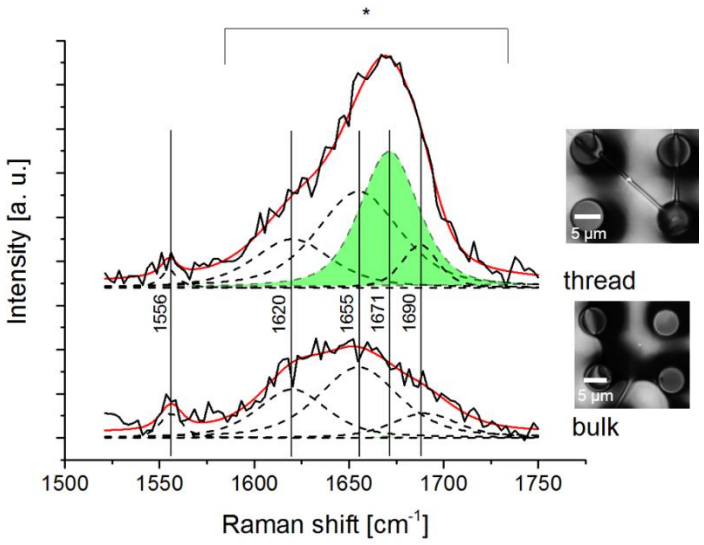

Fig. 3: Raman spectra collected on the thread bridging the pillars (thread) and on the collapsed drop (bulk). The bracket with asterisk indicates the region of interest of the Amide I for the protein secondary structure assignment. The black continuous line indicates the experimental spectrum, while the red line denotes the fitted spectrum. Dashed lines indicate the different Gaussian components of the band fittings, the one corresponding to the band of interest peaked at 1671 cm^{-1} is highlighted in green. Solid vertical lines identify the Raman shifts of the significant peaks localized by the band fittings. The optical images in the inset pictures exemplify a Raman measurement performed over a thread (top) and over an area of the collapsed drop (bulk-bottom). For each image the bright spot is the laser light focused in the area of interest.

Conclusion

Tau-related pathologies leading to neurodegeneration and cognitive impairment are still poorly understood. Here, we suggest that the combination of shear stress, laminar flow and crowded environment, which exist in the interstitial fluid and blood vessels of the brain could trigger the formation of toxic species in the form of tau aggregates. To mimic the brain environment we used a super-hydrophobic micro-patterned surface able to restrain the liquid solution into a confined thin volume in the shape of a capillary. Owing to the characteristics of the device, this was obtained by simple drop casting of a tau solution on its surface. The evaporation of the drop then, induces the formation of a liquid capillary tube where the micro-volume is limited by the air-liquid interface of the capillary only. The flow of the solution is laminar inside the capillary and increased shear stress, coupled with concentrated solute is present. Thus, the de-wetting on the pillared surface organizes the molecules in the liquid capillary and induces the formation of threads of tau bridging the pillars. By using the non-destructive Raman

spectroscopy technique, we successfully demonstrated that the thread bridging the pillars contains a significant percentage of β-sheets, distinctive of amyloid-like aggregation, as compared to an area of the collapsed drop where no structured organization of tau was detected. We therefore conclude that the shear flow generated by restraining of fluid in a confined environment is enough to re-organize the tau protein secondary structure without the need of additional crowding agent. Hence, we suggest that this mechanism could be involved also in the confined areas of the brain where a laminar flow is present, not only for the aggregation of tau protein, but also for any other IDP protein and prion-like protein.

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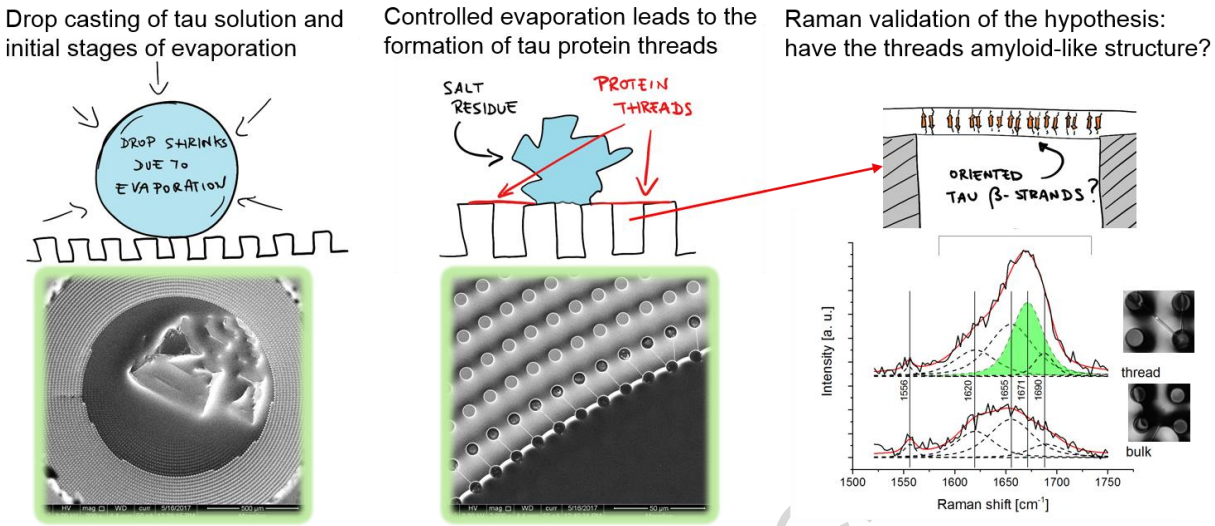
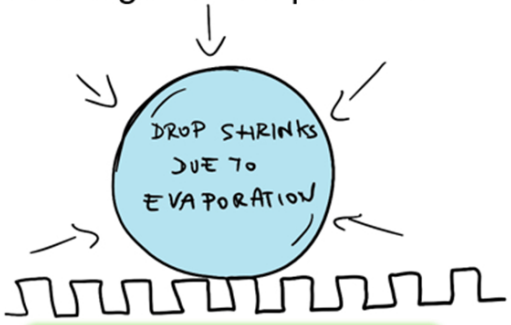


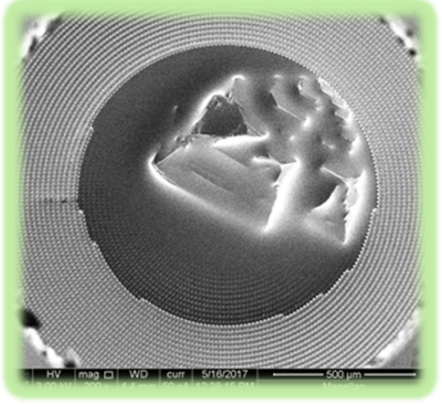
Graphical abstract

Highlights Ms. Ref. No.: MEE-D-17-00639

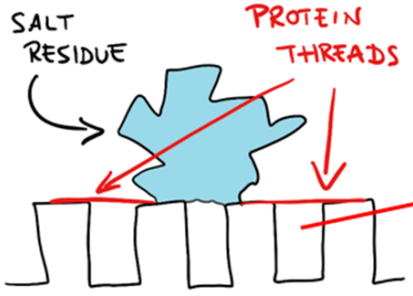
- Tau protein causes neurodegeneration: is laminar flow involved in its aggregation?
- A super-hydrophobic micro-patterned device is used to simulate the laminar flow
- A tau protein drop evaporates forming liquid capillaries across the pillars
- The capillary laminar flow drives the tau protein aggregation in amyloid-like form
- The β-sheet peak in the thread Raman spectrum confirms the amyloid presence

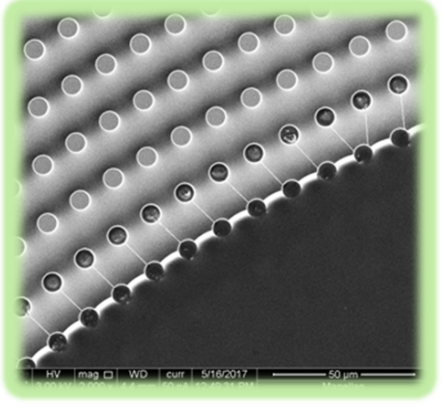
Drop casting of tau solution and initial stages of evaporation



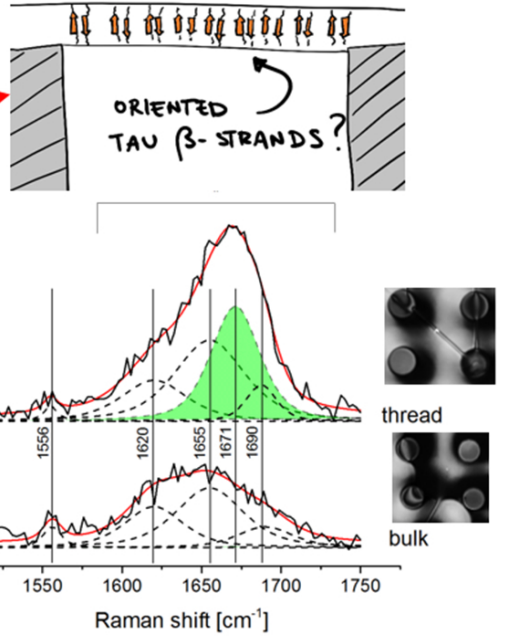


Controlled evaporation leads to the formation of tau protein threads





Raman validation of the hypothesis: have the threads amyloid-like structure?



Graphics Abstract

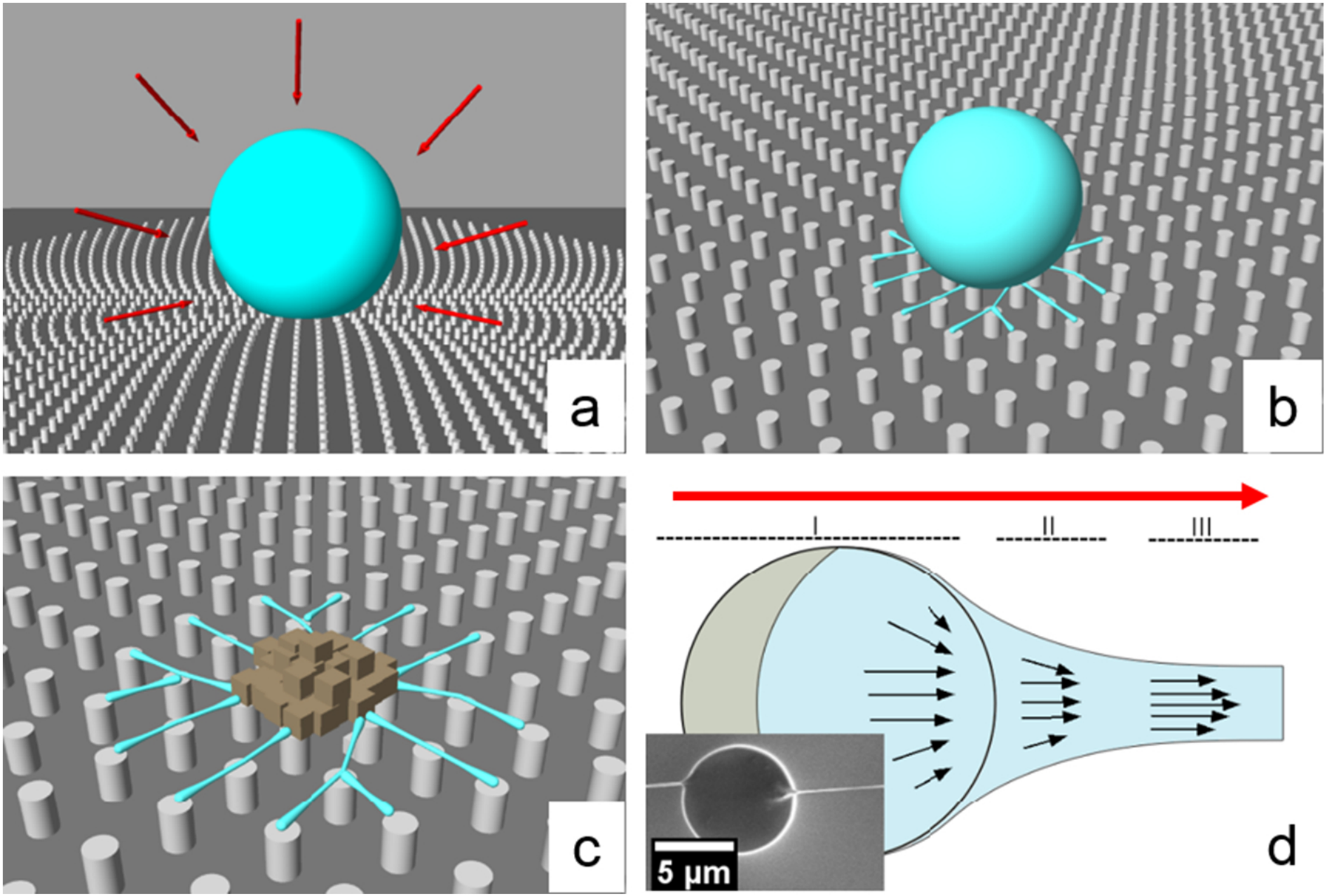


Figure 1

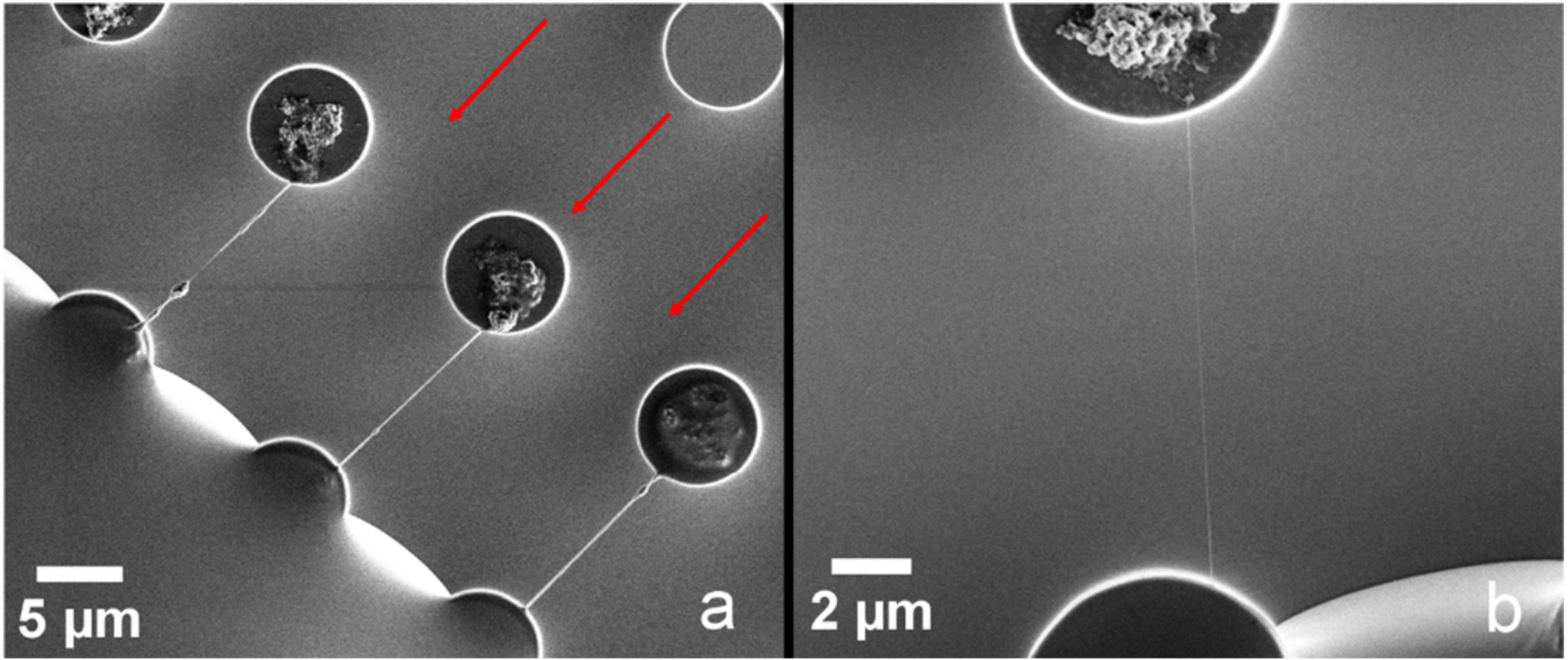


Figure 2

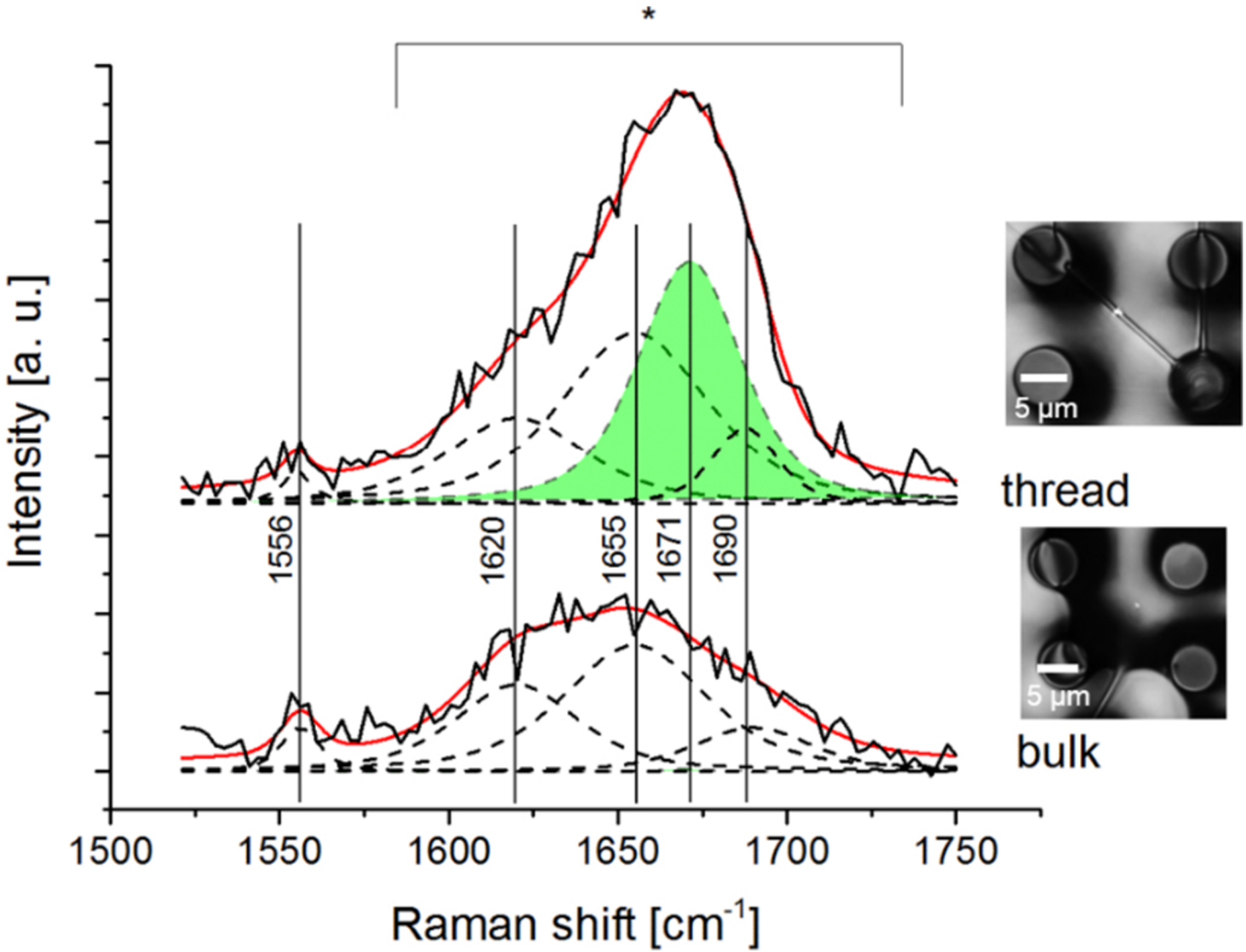

*
Intensity [a. u.]
1556
1620
1655
1671
1690
thread
bulk
5 µm
5 µm
1500
1550
1600
1650
1700
1750
Raman shift [cm-1]

Figure 3