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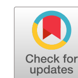
Research paper

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.

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1. 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 phosphorylated 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

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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.

2. Materials and methods

2.1. 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 3 × 3 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° and hence with a diameter of the footprint on the sample of ~1.5 mm.

2.2. 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, 150 mM NaCl, 0.25 mM DTT, 0.1 mM PMSF, 25% glycerol at −80 °C. The unwanted aggregates were spun down at 20,000*g* for 10 min before usage. The supernatant 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 μm 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 °C for the first 5 h and then was increased at 10 °C for 17 h. Afterwards the RH in the chamber was lowered to 50% for 5 h. 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.

2.3. 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 min. 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 3 kV and 50 pA current.

2.4. 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 °C 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 s 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.

3. 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 amino acids 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

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