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Research paper

Raman study of lysozyme amyloid fibrils suspended on super-hydrophobic surfaces by shear flow

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ABSTRACT

The shear flow generated at the rim of a drop evaporating on a micro-fabricated super-hydrophobic surface has been used to suspend and orient single/few lysozyme amyloid fibrils between two pillars for substrate-free characterization. Micro Raman spectroscopy performed on extended fibers evidenced a shift of the Amide *I* band main peak to the value attributed to β-sheet secondary structure, characteristic of the amyloid fibers. In addition, given the orientation sensitivity of the anisotropic molecule, the Raman signal of the main secondary structure was nicely enhanced for a fiber alignment parallel to the polarization direction of the laser. The substrate-free sample generated by this suspending technique is suitable for other structural analysis methods, where fiber crystals are investigated. It could be further employed for generation of arrays and patterns in a controllable fashion, where bio-compatible material is needed.

1. Introduction

Micro-patterned super-hydrophobic surfaces (SHS) are an ideal platform for the manipulation and characterization of single biological molecules and cells in background free environment [1–5]. The key element for suspending long molecules between two adjacent pillars is the shear flow generated at the rim of the evaporating drop [6]. This allows the single molecules to extend across the pillars top, ready for analysis. For example, literature reports DNA and DNA-protein complexes stretching over the micro-pillars substrates using this method, providing suitable samples to be analyzed by different techniques such as scanning electron microscope (SEM), transmission electron microscope (TEM) and Raman Spectroscopy [7–10]. In essence, when a drop of the solution containing the molecules of interest is deposited over this kind of super-hydrophobic surface, it will shrink until a residue accumulates at its center. Through the process, suitably long molecules might pin at the edge of the pillars, and due to the evaporation, they are pulled until they bridge the adjacent pillars in the direction of the flow. This is possible because the shape and design of the micro-fabricated surface maintain the drop quasi-spherical while shrinking, favoring the hopping of the receding meniscus from one pillar's edge to the next. Importantly, by structuring the surface it is possible to spatially organize the stretched molecules.

The three dimensional structure of a functional protein has a central role for its activity in the cellular environment and is characterized by the co-presence of three main types of secondary structure, namely α-helix, random coil, and β-sheet. Several pathologies are associated with the disarrangement of the tertiary structure of the protein, for example the diseases related to amyloidosis [11]. Amyloid deposits are characterized by the presence of highly oriented fibers made-up of β-strands organized into β-sheets and further into β-helix or solenoids, generated from the aggregation of misfolded or unfolded proteins which escaped the cellular quality-control [12]. The presence of β-sheet secondary structure can be confirmed by micro Raman spectroscopy, which is a non-destructive, label free technique especially suited for the localized biochemical analysis of biological samples. Appreciably, the results of Raman analysis can complement structural data obtained with other techniques such as NMR and x-ray spectroscopy, their most significant limitation being size (<40 kDa) and the need for single crystals, respectively [13].

In Raman spectroscopy, the Amide *I* band position, largely shifting around 1650 cm^{-1}, is routinely used to assign the α-helix, random coil, or β-sheet composition of the proteins. The recent literature agrees in assigning to the β-sheet secondary structure a Raman shift in the range 1665–1675 cm^{-1} [14,15]. An upshift of the Amide *I* band is evidenced in the transition from functional protein to amyloid structure, where α-synuclein and insulin were considered [16,17]. In 2007, a first report on functional amyloids derived from different types of silk fibers, highlighted the potential of polarized Raman spectroscopy, while

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assigning to the β-sheet secondary structure the Raman shift value in the range of 1666–1670 cm^{-1} [18]. They observed that the spectra were dominated by the contribution of the β-sheet, and used the peak height of the polarized Amide *I* band collected in different scattering geometries to demonstrate that the main Raman tensor axis of the Amide *I* is highly oriented perpendicular to the fiber direction. A more recent polarized Raman spectroscopy study takes into consideration insulin amyloid fibers aligned on a planar substrate by the coffee ring stain effect [19,20]. There, an increased intensity of the Raman peak centered at 1672–4 cm^{-1} emerges in the Raman spectrum when the fiber is aligned parallel to the laser polarization, an indication of the cross-β structure of the fiber core.

Here we verify by micro Raman spectroscopy the shift to a predominant β-sheet composition of the hen egg white lysozyme (HEWL) after its conversion to amyloid fibrils. The samples were prepared suspended across the gaps between neighboring pillars, and thus in a substrate and debris free environment. In addition, and for each suspended singular fiber, we observe a clear dependence of the intensity of the peak assigned to the β-sheet secondary structure to its orientation with respect to the exciting laser polarization, with a three-fold counts increase when oriented parallel to the laser polarization as compared to the perpendicular case. These results remark the ability of this technique to align single molecules along one given direction. Fiber suspension by shear flow on super-hydrophobic surfaces provides a fast and reproducible mean for the characterization of single molecules, purified form the solution buffer and debris, which typically represent a serious problem in standard preparation for laboratory analysis. The coupling of this preparation technique with Raman spectroscopy for the analysis of the single suspended fiber is then a powerful combination for the study of any anisotropic fiber crystal, where the structural characterization is not trivial by means of, e.g., x-ray crystallography or NMR.

2. Materials and methods

2.1. Super-hydrophobic surface fabrication

Super-hydrophobicity emerges as a result of the patterning and functionalization of the surface, whose combination provides very high contact angle displayed by these substrates (>150°). In this case, the pattern is a sequence of concentric circular arrays of pillars with radial pitch of 18 μm, each of the pillars having a diameter of 6 μm and height of 7 to 10 μm. To maintain a uniform surface coverage, the number of pillars in each circular array was increased according to the recursive formula $N_i = N_{i-1} + 4$, where N_i is the number of pillars of the *i*th circle. The patterning of the surface was obtained with a combination of optical lithography and deep reactive ion etching (DRIE) technique. The original substrate was a 4″ standard Si ⟨100⟩ wafer. In the first step, the pillars pattern was defined by means of negative optical lithography. The sample was then etched by means of DRIE (PlasmaLab System 100, Oxford Instr.), with a final height of the pillars of about 10 μm. Finally, the functionalization of the surface with hydrophobic material was achieved by the deposition of Perfluorodecyltrichlorosilane (FDTS) in a Molecular Vapor Deposition System (MVD100E, Applied MST). FDTS in the presence of water reacts with OH groups which are exposed at the surface of the oxidized Si, creating a monolayer of hydrophobic fluoro-carbon chain molecules bound to the sample surface. The functionalizing FDTS was dispensed together with water vapor in a vacuum pumped chamber of an MVD commercial system (MVD100E, Applied MST).

2.2. Lysozyme fibrils preparation

Lysozyme amyloid fibrils were produced starting from a solution of HEWL powder (Sigma) [10 mg/ml] in MilliQ water based on [21]. Acidification of the solution to pH ~ 2.0 was obtained by adding 9% (*v*/v) of HCl [1 N] in the solution. The mix was then immersed in a water bath at 60 °C for 120 h, until the formation of suitably long amyloid fibrils, as verified by AFM. No purification of the amyloid fibers was performed. The solution was then diluted ten times before depositing a 10 μl drop over the super-hydrophobic substrate with a hypodermic syringe microneedle. The drop was, thereafter, dried at room temperature and 50% humidity for 3 h.

2.3. Sample characterization by AFM and SEM

The formation of amyloid fibrils at least 12 μm long (since this is the exact distance between the pillars edge in radial direction) was monitored by AFM. A 30 μl drop was withdrawn every 12 h and spotted on a freshly cleaved mica sheet. It was let adsorb for 10 min and then rinsed thoroughly with MilliQ water. Finally it was N_2 dried and ready for measurement. JPK Nanowizard III mounted on inverted Olympus IX73 microscope was used for the measurement at relative humidity below 15%. XSC11 AFM probes (MikroMasch, Nanoworld AG) with nominal resonance frequency of about 150 kHz and nominal force constant of 7 N/m were run in tapping mode for the topography measurement.

After drop-casting the solution over the pillars top, we let it dry for 3 h, then the deposition of the fibers was verified by a Quanta 200 FEG Scanning Electron Microscope (FEI) at 5 kV beam voltage and 64 pA current. Before imaging, the sample was sputter coated with 2 nm of Iridium, to avoid charging effects.

2.4. Raman spectroscopy of lysozyme amyloid fibrils

Laser micro-Raman spectroscopy was performed in confocal back scattering geometry by exciting the sample with 532 nm linearly polarized laser wavelength (Coherent Compass Sapphire Laser, 75 mW) at 4 mW power on a WiTec Raman spectrometer (Alpha300 RA) with Andor CCD detector (DU970N) cooled at −65 °C and a 100× objective (Zeiss, EC EPIPLAN NEOFLUAR, 0.9 NA). Measurements of the lysozyme powder dissolved in MilliQ water and lysozyme fibrils in acidic solution were performed after depositing the sample on a CaF_2 substrate to minimize signal background. The range of pillars height and spacing useful for effective deposition is chosen to be bigger than the focal length and lateral dimension of the focus of the objective. In this way the excitation volume is optimized to minimize the background from the silicon substrate. For the measurement of the fiber anisotropy, the sample was oriented at 0° (parallel) or 90° (perpendicular) with respect to the polarization of the excitation laser. Data analysis was carried out as follows: at least ten measurements per sample were acquired, baseline subtracted in the range 1100–1800 cm^{-1} (by grade 5 polynomial fitting) and averaged. Afterwards all the spectra were normalized to the Raman peak intensity at 1450 cm^{-1} to compare data. Amide *I* spectral region was fitted to a band described by a mixture of Lorentzian and Gaussian functions.

3. Results and discussion

3.1. Amyloid fibers on SHS

Lysozyme amyloid fibrils suspended between pillars of a super-hydrophobic surface bear four major characteristics that make their study by Raman spectroscopy unique: they are made of single/few isolated molecules, free from the presence of a substrate, clean and not contaminated by debris, and highly oriented along one given direction. This effect is obtained by the shear flow generated in the receding meniscus of the drop which contains the reaction solution (including fibrils, oligomers and native enzyme) and is evaporating over the micro-patterned SHS, and it was first demonstrated by De Angelis et al. [1]. In this work, cylindrical micro-pillars arranged in a circular pattern were used. Pillars inter-distance was 12 μm. The result of the dehydration process can be appreciated in Fig. 1: in Fig. 1a, four different drying areas emerge as a result of the evaporation of the drop