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Superhydrophobic Surfaces Boost Fibril Self-Assembly of Amyloid β Peptides

Angelo Accardo[a,], Victoria Shalabaeva[a], Emanuela Di Cola[b,1], Manfred Burghammer[b], Roman Krahne[a], Christian Riekel[b] and Silvia Dante[a]*

[a] *Istituto Italiano di Tecnologia, Via Morego 30, Genova 16163, Italy*

[b] *The European Synchrotron, CS40220, 38043 Grenoble Cedex 9, France*

**Corresponding author's e-mail address: angelo.accardo@iit.it*

[1]*Current address: Laboratoire Interdisciplinaire de Physique (LIPhy UMR5588 CNRS/UJF), 140 rue de la Physique, BP87 38402 Saint Martin d'Hères Cedex.*

KEYWORDS: Superhydrophobic, Convective flows, β-amyloid, Fibril self-assembly, X-ray diffraction, Synchrotron radiation.

ABSTRACT

Amyloid β (Aβ) peptides are the main constituents of Alzheimer's amyloid plaques in the brain. Here we report how the unique microfluidic flows exerted by droplets sitting on superhydrophobic surfaces can influence the aggregation mechanisms of several Aβ fragments by boosting their fibril self-assembly. Aβ(25-35), Aβ(1-40) and Aβ(12-28) were dried both on flat hydrophilic surfaces (contact angle, CA=37.3°) and on nanostructured superhydrophobic ones (CA=175.8°). By embedding nanoroughened surfaces on top of highly X-ray transparent Si_3N_4 membranes, it was possible to probe the solid residues by raster-scan synchrotron radiation X-ray micro-diffraction (μXRD). As compared to residues obtained on flat Si_3N_4 membranes, a general enhancement of fibrillar material was detected for all Aβ fragments dried on superhydrophobic surfaces, with a particular emphasis on the shorter ones. Indeed, both Aβ(25-35) and Aβ(12-28) showed a marked crystalline cross-β phase with varying fiber textures. The homogeneous evaporation rate provided by these nanostructured supports, and the possibility to use transparent membranes, can open a wide range of *in-situ* X-ray and spectroscopic characterizations of amyloidal peptides involved in neurodegenerative diseases and for the fabrication of amyloid-based nanodevices.

Introduction

Amyloids are insoluble protein aggregates which are the main cause of more than twenty human diseases.[1,2] Among these amyloid-dependent syndromes are neurodegenerative pathologies such as Alzheimer's,[3] Parkinson's[4] and Huntington's diseases[5] but also non-neuropathic localized amyloidosis such as diabetes mellitus type II.[6,7] Several approaches and innovative techniques have been used in the last decades to monitor the mechanisms which lead to the formation of the fibrils that are at the base of the above mentioned diseases.[8-13] Considering how the number of patients affected by dementia will triple by 2050,[14] going from the actual 45 million to more than 135 million, the role of Alzheimer's disease (AD) assumes huge social implications. AD is characterized by the formation of extracellular amyloidal plaques, rich in fibrils of Aβ(1-40) and Aβ(1-42), which may act also as reservoir for the most toxic Aβ membrane-soluble species, such as oligomers and protofibrils.[15,16] A more detailed knowledge of the aggregation path differentiation for the various Aβ species and of the final structure adopted by their fibers can add new information about the role of the different Aβs in the neurodegenerative mechanisms.

Our aim is showing that amyloid fibrillation mechanisms can be sensitively affected by the presence of superhydrophobic and superhydrophilic surfaces and materials.[17-20] Indeed, molecular self-assembly has been demonstrated for a wide range of materials and mechanisms of biological interest[17] such as synthetic amyloid peptides,[21,22] protein conformational changes,[18,23] biomineralization processes,[24] nanocrystallization,[25] DNA pulling,[26] and healthy/tumoral cell exosomes discrimination.[27] For droplets on hydrophilic surfaces, the evaporation rate is highest at the triple contact-line resulting in an outward convective flow, while droplets on superhydrophobic surfaces (SHSs) have a more homogeneous evaporation rate across their surface resulting in the presence of a circulatory convective flow.[17] The flows generated inside

evaporating drops on SHSs can then be used to induce ordering effects in the analyte as observed for the formation of fibrillar lysozyme related to a local convective flow field developed during pinning of the drying drop at the interface between the droplet and the substrate.[28] Further, synthetic trimeric to hexameric peptides show residues with marked cross-β sheet configuration after evaporation of quasi contact-free droplets on superhydrophobic surfaces.[21,22] Under the same conditions, lysozyme in presence of Ca^{2+} ions and a weakly acidic environment, self-assembles also into a fibrillar amyloidic phase on a time scale much shorter than that reported in literature.[23] On the other hand, several Aβ fragments, which are directly involved in the formation of AD plaques in the brain, show a direct correlation between secondary structure conformational changes, presence of nanostructured surfaces and peptide concentration.[18,19] Besides amyloidal proteins involved in different pathologies, there is also a class of self-assembling peptides which, thanks to their low immunogenic and inflammatory potential, are at the center of a fascinating research branch which aims at different applications in the field of nanobiotechnology.[29,30] Among these "functional amyloids" are prion-related ones which are essential for the storage of memory in the brain,[31] amyloid fibers that serve as templates for the biosynthesis of melanin[32,33] and amyloid fibrils employed as biomaterial for promoting cell adhesion, migration and differentiation.[34] Considering all these aspects, the existence of a protocol able to control the formation of fibril-self assembly in such bio-compounds would be crucial not just for better understanding the formation of amyloid material in neurodegenerative and non-neurodegenerative diseases, but could also be exploited as a versatile fabrication approach of novel biomaterials for biomedicine and tissue engineering purposes.

The ability to manipulate biological soft matter aggregation has been exploited in this work to guide the process of fibril self-assembly in Aβ proteins by the peculiar microfluidic flows

induced by biomimetic SHSs, which mimic lotus leaves superhydrophobic features. For this aim we embedded poly(methyl methacrylate) (PMMA) nanostructuresd SH coatings on ultrathin Si_3N_4 membranes in order to perform *in-situ* X-ray micro-diffraction (μXRD) studies on the dried residues. Following this approach we made a comparative evaluation of different amyloid β-peptides, namely Aβ (25-35), Aβ (1-40) and Aβ (12-28), confined in droplet environments both on flat hydrophilic Si_3N_4 membranes and on SH ones. While on hydrophilic supports it is possible to observe characteristic ring-like moieties already studied elsewhere,[18,19,35] the homogeneous evaporation rate provided by superhydrophobic surfaces[17] induce the formation of freestanding samples. As compared to flat Si_3N_4 membranes, Aβ fragments in presence of superhydrophobic supports result in a strong enhancement of the fibrillar component, often accompanied by a quasi-crystalline structure. We show that raster-scan diffraction with a micrometric synchrotron radiation X-ray beam is required for revealing local super-structures with variable fiber textures. The control over the aggregation mechanisms of fibril self-assembly in natural peptides, provided by the use of the protocol presented in this study, is a valuable tool both to shed more light on the processes resulting in the formation of amyloid fibrils in neurodegenerative diseases and as a fabrication route for the development of amyloidal films to be used in the field of biomedicine. In this work we present a new class of superhydrophobic X-ray transparent Si_3N_4 membranes allowing to investigate the effects of convective flows in μL droplets on the self-assembly mechanisms of amyloid-β peptides involved in Alzheimer's disease by μXRD.

Experimental section

Aβ Peptides. Aβ(1-40) (H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-

Gly-Gly-Val-Val-OH, molecular formula $C_{194}H_{295}N_{53}O_{58}S$), Aβ(12-28) (H-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-OH, molecular formula $C_{89}H_{135}N_{25}O_{25}$) and Aβ(25-35) (H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH, molecular formula $C_{45}H_{81}N_{13}O_{14}S$) fragments were products of Bachem. The peptides were first dissolved in trifluoroacetic acid (TFA) at a concentration of 1 mg/mL to eliminate the presence of seeds and to obtain a monomeric peptide dispersion. TFA was dried under nitrogen, and the peptides were successively dissolved in Milli-Q water (1 mg/mL), shortly sonicated, and centrifuged at 10000 RPM for 10 min.

Superhydrophobic Si_3N_4 membranes fabrication. Si_3N_4 – Si – Si_3N_4 <100> wafers (Si thickness 525 μm, Si_3N_4 thickness 500 nm) were purchased from University Wafer. We adopted an optical lithography process in order to obtain a matrix of single square Si_3N_4 membranes of 5 mm x 5 mm size. After spin-coating the wafer with S1813 positive resist at 4000 RPM for 60 s, the wafer went through a baking process (90°C for 3 minutes). UV exposure was performed with a SUSS MicroTech mask-aligner in soft contact for 15 seconds and it was followed by a MF319 development for 1 minute. A reactive ion etch process (Sentech 500 system, CHF_3 70 sccm, O_2 5 sccm, RF 70 W, ICP 200 W, P = 1 Pa) allowed to etch the Si_3N_4 regions not covered by the optical resist. The residual resist was then stripped by cold acetone (5 min) and the entire wafer passed through a wet etch based on KOH (100 g of KOH in 150 ml of H_2O at 105°C) for 6-8 hours. After etching the Si, the wafer was rinsed in milliQ water and baked at 65°C to dry it completely before performing the superhydrophobic coating. The wafer was spin-coated with PMMA A11 (MicroChem) resist at 2000 RPM for 60s and baked (180°C for 30 min) obtaining an homogenous layer of 1 μm thickness. A further plasma reactive ion etching process was employed to nanotexture the PMMA layer (ICP 50 W, RF 100 W, P = 6.5 Pa, O_2 30 sccm, 5

min) and to deposit a thin layer of Teflon on top of it (RF 600 W, P = 3.6 Pa, C_4F_8 85 sccm, 5 s).

Contact Angle measurements. The evaluation of aqueous droplets contact angle was estimated using a Kruss DSA100 setup, yielding a value of 37.3° ± 0.5° in presence of flat pristine Si_3N_4 supports and 175.8° ± 0.5° in presence of superhydrophobic Si_3N_4 supports (Supporting Information, Figure S1).

Synchrotron Radiation Experiments. μXRD experiments were performed at the ID13 Microfocus beamline of the European Synchrotron Radiation (ESRF). A monochromatic beam of λ = 0.0968633 nm was focused to a 2.5(v) × 2.8(h) μm^2 with about 3×10^{11} photons/s flux at the sample position using a transfocator[36] based on parabolic Be-refractive lenses. A Frelon CCD detector with 2048×2048 pixels of 50 × 50 μm^2 each (binned to 4×4) was used for data collection with an exposure of 0.5 s per pattern. The sample-to-detector distance was evaluated to be 142.1 mm by an Ag-behenate calibrant. Regions of interest of the sample were selected using an on-axis optical Olympus microscope aligned with the focal spot of the microbeam. The expression of the scattering vector Q in the illustrated azimuthal averages is equal to $Q = 4\pi \sin\theta/\lambda$, where θ is the diffraction angle and λ is the X-ray wavelength. FIT2D and an ID13 beamline in-house developed software have been used for data analysis.

Scanning Electron Microscopy (SEM). SEM images were recorded by a FEI HELIOS Nanolab 600 instrument with a 10 kV acceleration voltage on samples sputtered with a 10 nm layer of gold.

Results and discussion

Figure 1A illustrates the typical experimental configuration of the μXRD experiments. After evaporation of 5 μL droplets of Aβ aqueous solutions (1 mg/mL) either on the superhydrophobic

Si_3N_4 membranes (Figure 1B,C) or on the flat pristine hydrophilic ones, the windows were mounted on a goniometer in front of a highly collimated X-ray beam to perform μXRD analysis (see Methods, Figure 1A and Figure S1) in transmission geometry exploiting the X-ray transparency of the membrane. In all the experiments that we performed we evidenced a sharp difference between the residues obtained on superhydrophobic and hydrophilic supports. On the latter ones we obtained ring-like residues very adherent to the surface (Figure 2A-4A) confirming how the evaporation flux configuration on a hydrophilic surface induces an outward convective flow, a pinning of the droplet and the formation of a coffee-ring residue.[35]

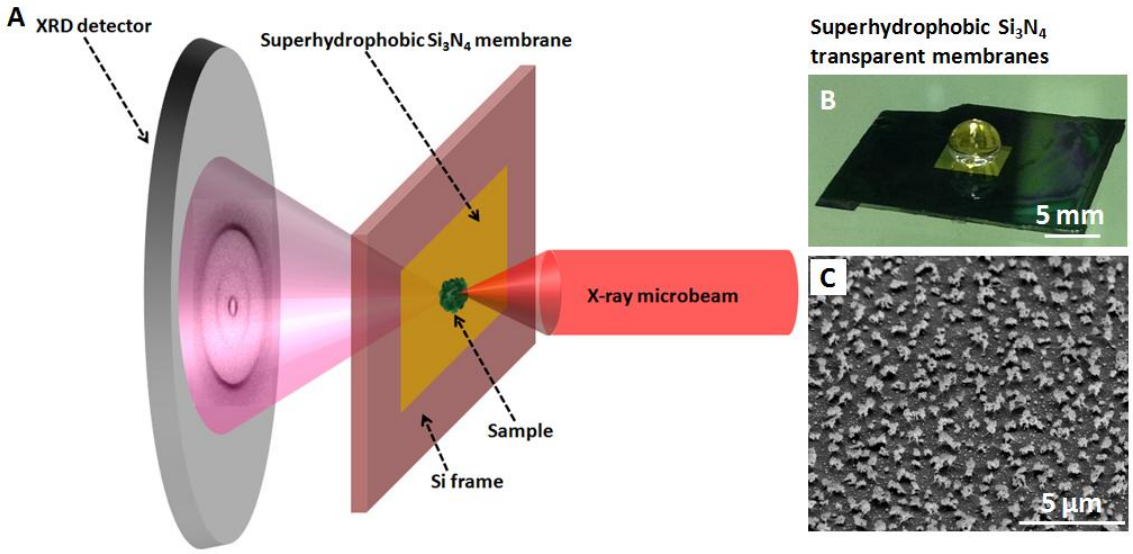

Figure 1 A: Sketch of the experimental setup in the ID13 microfocus beamline with the X-ray beam impinging the sample formed over the superhydrophobic membrane and resulting in a scattering pattern recorded by the XRD detector (the experimental environment of the beamline is shown in figure S1); B: Aqueous droplet sitting on a superhydrophobic Si_3N_4 membrane; C: SEM micrograph evidencing the rough morphology of the PMMA coating on the superhydrophobic Si_3N_4 membranes.

On the contrary, the presence of recirculating convective flows, of an extremely homogenous evaporation rate and of a low interaction between the droplet and the underneath superhydrorepellent surface[17] induced the formation of free standing tridimensional residues (Figure 5A-7A).

μXRD analysis of the Aβ peptides dried on flat Si_3N_4 membranes.

Figure 2A shows the optical image of a typical ring-like residue obtained from Aβ(25-35) peptide dried on a flat Si_3N_4 membrane. The inset shows the morphology of the external rim while Figure 2B illustrates a XRD mesh scan of such region where each single "pixel" is a XRD pattern covering a 2.5(v) × 2.8(h) μm^2 area according to the beam spot-size (see Methods).

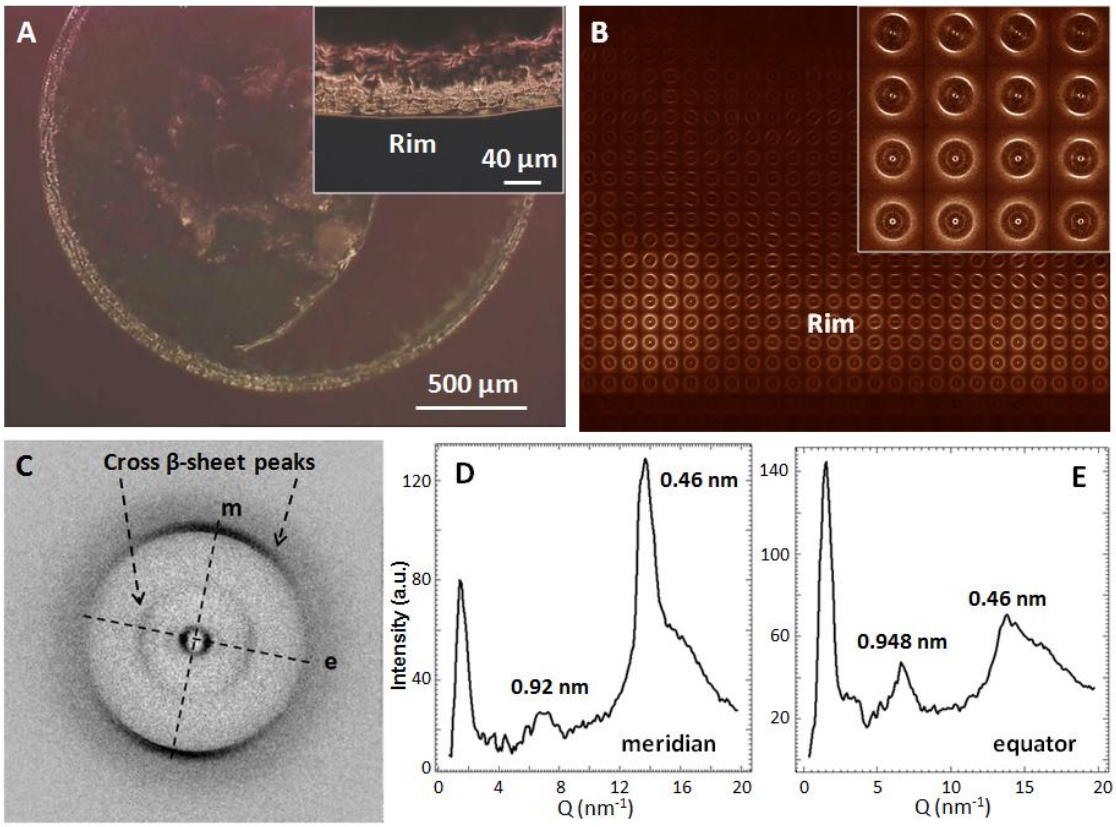

Figure 2 A: Optical image of Aβ(25-35) ring-like residue dried on a flat Si_3N_4 membrane (inset: close-up on the rim of the residue); B: μXRD mesh scan of a portion of the rim in figure 2A (inset: close up on a few μXRD patterns); C: Typical orthogonal μXRD pattern from the rim of the residue highlighting the meridional and equatorial directions; D,E: Azimuthal averages of the μXRD pattern of Figure 2C along the meridian (D) and equatorial (E) directions.

This peptide has been extensively studied in previous works[18,19] on superhydrophilic surfaces where it showed a concentration-dependant α-helical to β-sheet transition. As shown by a typical XRD pattern of the rim (Figure 2C), in presence of a conventional hydrophilic surface, such as flat Si_3N_4, we have the presence of a distinct cross-β sheet structure exhibiting a meridional d =

0.46 nm spacing (azimuthal average in Figure 2D), related to the distance between hydrogen-bonded strands, and an equatorial spacing of 0.948 nm (azimuthal average in Figure 2E), characteristic of β-sheet stacking. This configuration is universally recognized as the gold standard to unmistakably define amyloid fibrils.[2]

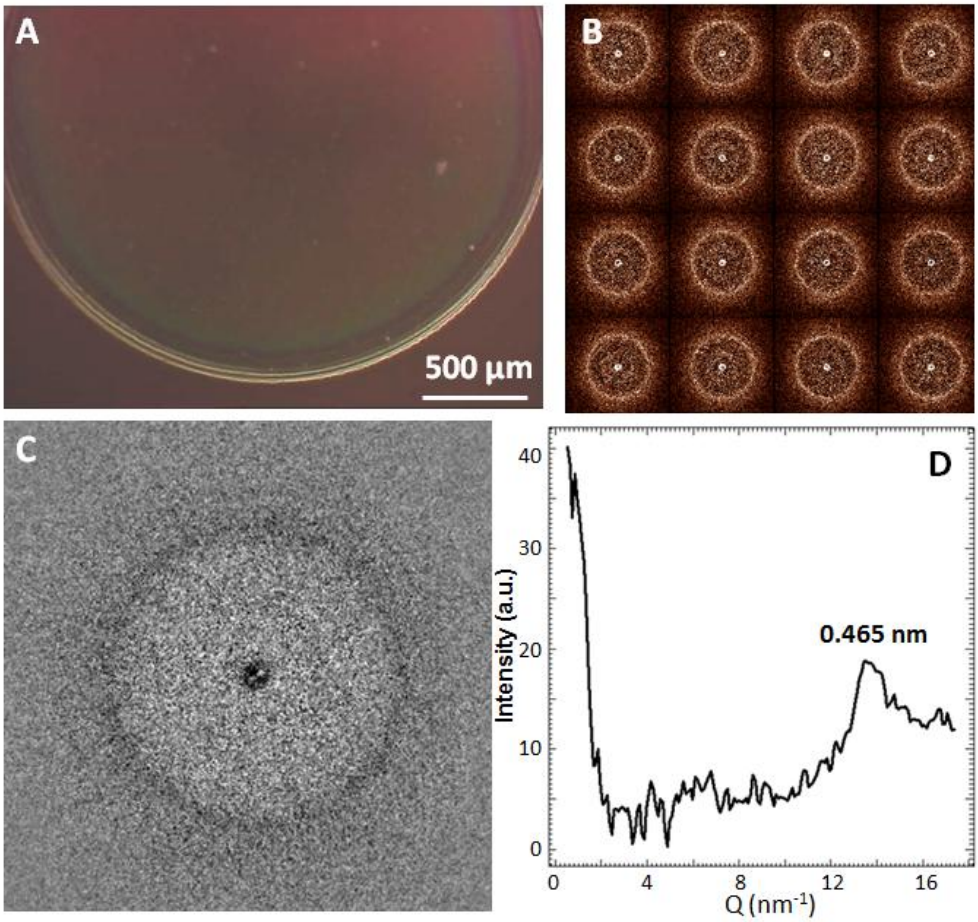

Figure 3 A: Optical image of Aβ(1-40) ring-like residue dried on a flat Si_3N_4 membrane; B: µXRD mesh scan of a portion of the rim in figure 3A; C: Typical µXRD pattern from the rim of the residue; D: Azimuthal average of the µXRD pattern of Figure 3C.

As demonstrated in previous works, the kinetics and amyloid morphology of Aβ peptides can be significantly different.[37] As further confirmation of these differences, Aβ(1-40) peptide showed a very different structure on flat supports if compared with the Aβ(25-35) one. Although the morphology of the residue looked very similar to the one of Aβ(25-35) (Figure 3A), the typical XRD patterns (Figure 3B,C) revealed only an unordered β-type phase with a weak contribution

at d ≈ 0.465 nm (Figure 3D), related to hydrogen-bonded strands. A similar configuration was found for the fragment Aβ(12-28) (Figure 4A-E) even though in this case we detected a quite sharp fibril orientation (Figure 4C).

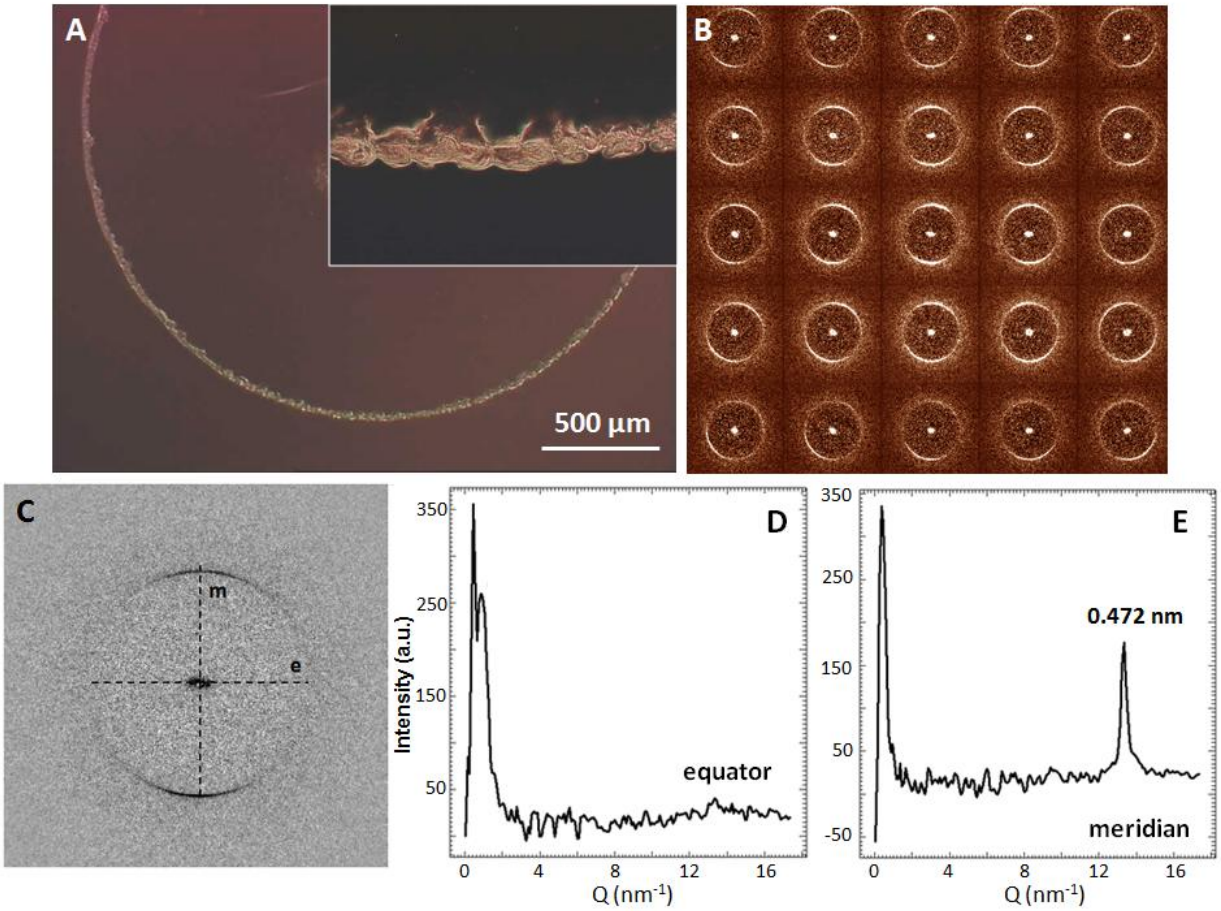

Figure 4 A: Optical image of Aβ(12-28) ring-like residue dried on a flat Si_3N_4 membrane (inset: close-up on the rim of the residue); B: µXRD mesh scan of a portion of the rim in figure 4A; C: Typical µXRD pattern from the rim of the residue highlighting the meridional and equatorial directions; D,E: Azimuthal averages of the µXRD pattern of Figure 4C along the equatorial (D) and meridian (E) directions.

As shown by the XRD mesh scan and the single XRD pattern, respectively, in Figures 4B,C, the rim showed several areas with marked β-sheet conformation with a strongly oriented meridional reflection at d ≈ 0.472 nm (Figure 4E). As happened for the Aβ(1-40) peptide, also in this case there was no appearance of the ≈1 nm reflection characteristic of β-sheet stacking, suggesting a possible stacking disorder.

It is worth noting that the aggregation kinetics of the investigated fragments, *i.e.*, their conversion from non-amyloidogenic to amyloidogenic forms, differs significantly. In particular, for Aβ(1-40), which in aqueous solution, at physiological conditions, has a partially unfolded structure,[38] fibrillogenesis is rather low and may occur in several hours, depending on its concentration;[37,39] on the contrary the undecapeptide Aβ(25-35), which is the shorter Aβ fragment retaining the toxic characteristic of the full length Aβ peptide, shows a very rapid self-assembly behavior.[40] These differences reflect distinct aggregation paths and possibly the presence of different intermediates, which may therefore aggregate in diverse molecular architectures, as indicated by our findings.

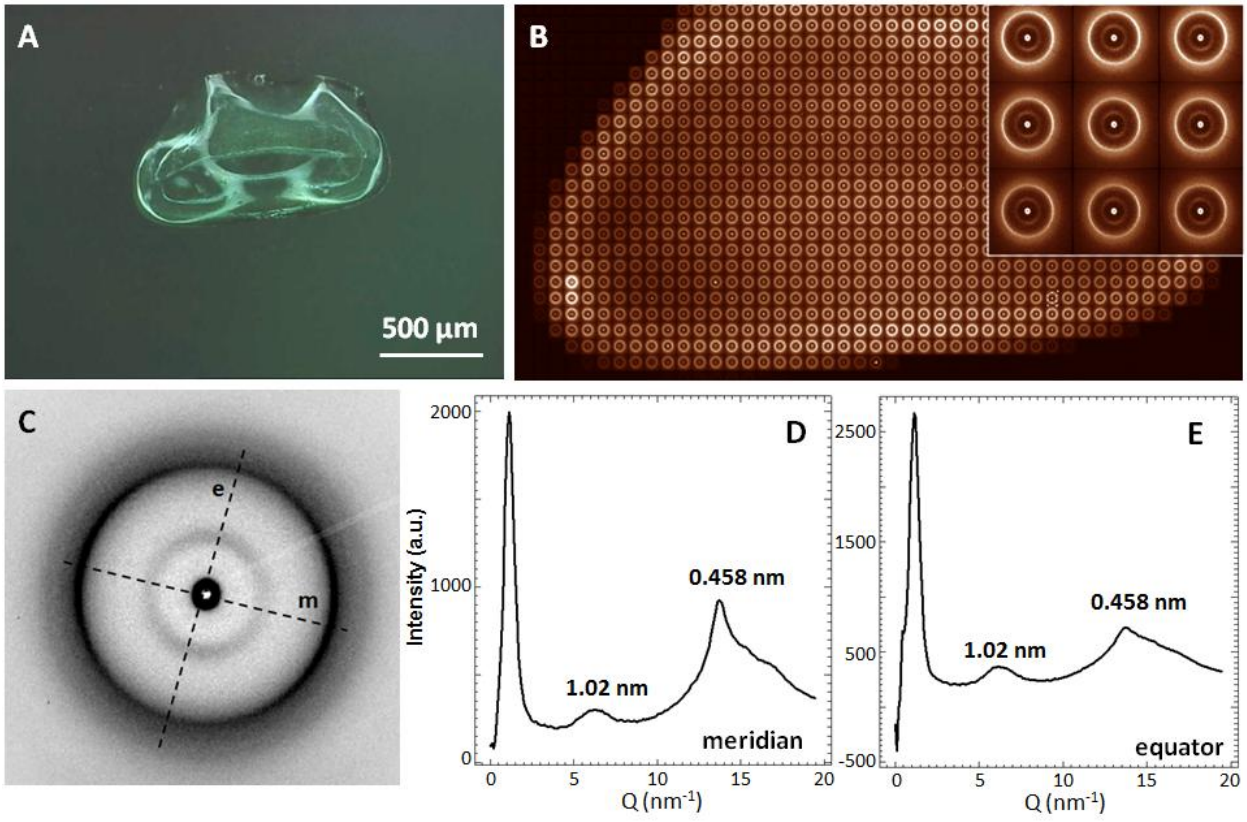

Figure 5 A: Optical image of Aβ(1-40) free-standing residue dried on a superhydrophobic Si_3N_4 membrane; B: µXRD mesh scan of the residue in figure 5A (inset: close up on a few µXRD patterns); C: Typical µXRD pattern of the residue highlighting the meridian and equatorial directions; D,E: Azimuthal averages of the µXRD pattern of Figure 5C along the meridian (D) and equatorial (E) directions.

μXRD analysis of the Aβ peptides dried on superhydrophobic Si_3N_4 membranes.

The conformation of the analyzed β-amyloid peptides changes abruptly in presence of the superhydrophobic X-ray transparent supports. The optical image in figure 5A shows a typical free-standing residue of a Aβ(1-40) solution droplet dried on the superhydrophobic surface. The μXRD raster-scan (Figure 5B) reveals a β-type fiber texture contrasting the weak unoriented powder-like β-type material obtained in the hydrophilic case (Figure 3). In particular the azimuthal averages along the equatorial and meridional directions (Figure 5D,E) of a typical XRD pattern (Figure 5C) extracted from the mesh scan, highlights the presence of an orthogonal cross β-sheet configuration with the characteristic reflections at 1.02 nm and 0.458 nm. This result shows how, on very short time scales (each evaporation of a 5μL droplet takes around 1 h), it is possible to boost the self assembly of amyloid fibrils of an Aβ peptide which, dried on conventional flat hydrophilic surfaces, does not show any ordered fibril structure. This can be attributed to the peculiar microfluidic flows and the homogeneous evaporation rate provided by droplets sitting on superhydrophobic supports that give enough time to the analyte to arrange in a gradual and more ordered way if compared with the hydrophilic substrate. In this latter case indeed the evaporation rate is very strong at the edge of the droplet and very low at the center, inducing a faster, but highly heterogeneous, evaporation rate which pushes the analyte to aggregate just at the rim of the solid residue.[18,19,35]

This "boosting" effect becomes even more evident with the shorter peptides under investigation. Figure 6A shows a XRD mesh scan of the Aβ(12-28) peptide for which we obtained a free-standing residue characterized again by highly oriented amyloid fibril patterns as better shown in Figure 6B,C. Albeit in presence of a hydrophilic support we already observed the presence of oriented patterns (Figure 4), in that case we could detect only the typical meridional

reflection at d ≈ 0.472 nm characteristic of hydrogen-bonded strands. On the contrary, in the superhydrophobic case it is possible to appreciate the presence of a very well defined region of the residue where the typical XRD patterns (Figure 6C) show a marked cross β-sheet configuration including, besides the d ≈ 0.467 nm reflection, also the orthogonal d ≈ 1.12 nm one related to β-sheet stacking (Figure 6D,E).

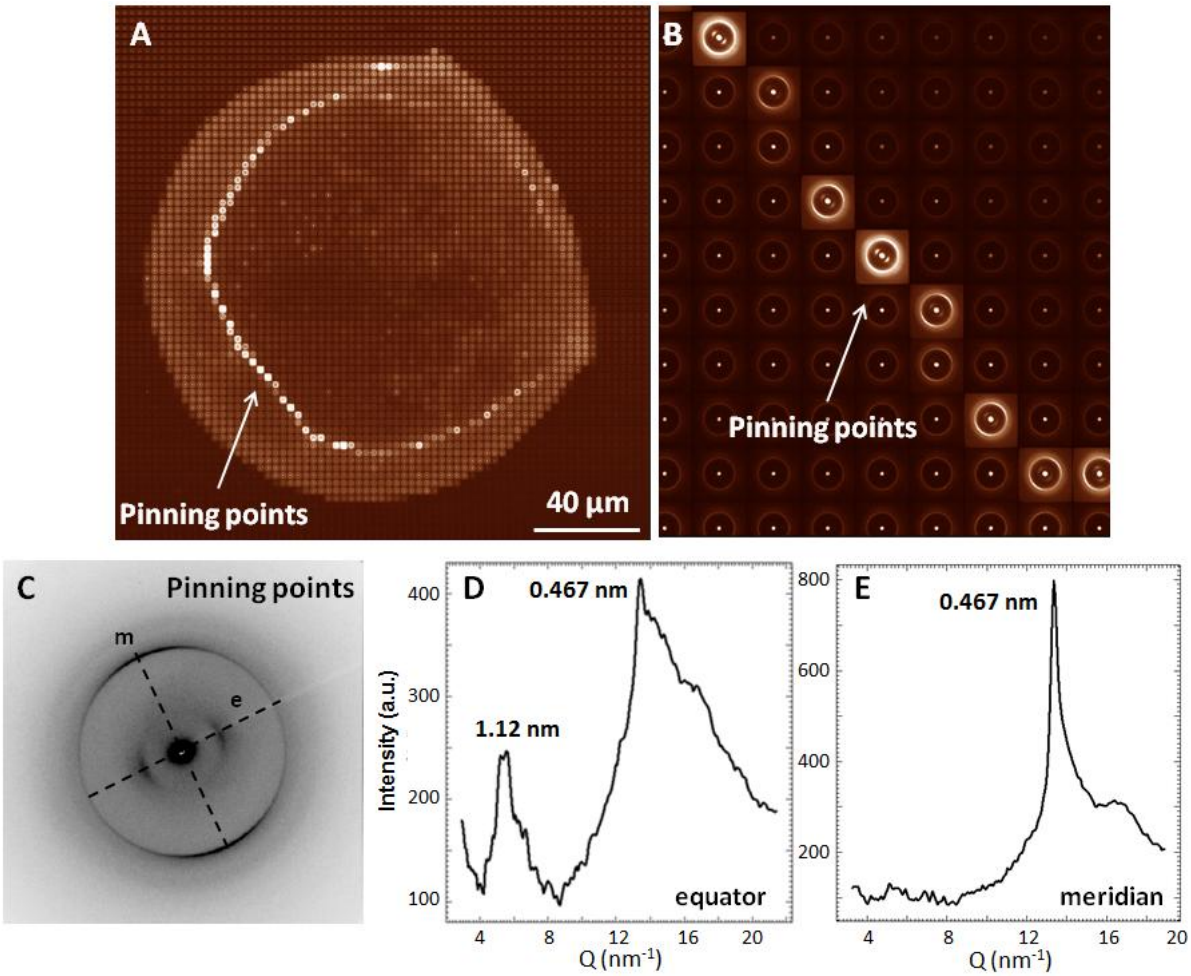

Figure 6 A: µXRD mesh scan of the Aβ(12-28) residue dried on a superhydrophobic Si_3N_4 membrane; B: close up on the µXRD patterns located in proximity of the droplet pinning points; C: Typical µXRD pattern of the residue highlighting the meridional and equatorial directions; D,E: Azimuthal averages of the µXRD pattern of Figure 6C along the equatorial (D) and meridian (E) directions.

The presence of these patterns mainly concentrated in a sort of "crown" is imputable to the pinning effect of the drying droplet as illustrated in a previous investigation of our group.[17,28] Indeed, after being deposited in a superhydrophobic state (*i.e.*, with a contact angle higher than

150°), during the evaporation process the droplet starts to shrink due to the volume reduction. According to the aspect ratio of the surface roughness (which sustains the droplet in a superhydrophobic state) and to the viscosity of the droplet itself (which depends on the contained analyte, in this case Aβ peptide), before the end of the evaporation process the droplet dimension becomes so small that it starts to pin the asperities, thus transiting from a superhydrophobic "suspended" state (Cassie state) to a hydrophobic "pinned" one (Wenzel state).[17,28] In this configuration the droplet is not anymore in a quasi contact-free state on the substrate and this can provoke some sensitive changes in the convective flows acting inside the droplet and in a partial dis-homogenization of the evaporation rate. In summary, after having exploited the homogeneous evaporation rate of the superhydrophobic state for almost the whole drying process (the pinning effect on SHSs like the ones used here, with a CA greater than 170°, happens only at the very end of the process), the ordered fibril material which formed during this time can get trapped in proximity of the pinning points (Figure 6A,B) and form the "crown" illustrated by the XRD mesh scan.

Finally, the apex of fibril boosting induced by the superhydrophobic membranes was reached by Aβ(25-35). Already in its ring-like conformation, obtained with hydrophilic supports, this fragment showed a peculiar predisposition to form amyloid fibers as illustrated in Figure 2. We managed separating a free-standing residue from the superhydrophobic membrane and attached it to a glass capillary for X-ray analysis (Figure 7A). A composite raster-scan image reveals that the rim of the residue is composed of highly oriented fiber diffraction patterns (Figure 7B). A highly structured fibrillar morphology can also be seen in scanning electron microscopy (SEM) images (Figure 7C-E). This contrasts powder-like patterns obtained by drying solubilized Aβ(25-35).[41]

The relatively large number of reflections exhibited by this Aβ fragment shows a para-crystalline order similar to the one detected also for other synthetic peptides[42] after 1 week incubation time, a far longer duration if compared with our droplet evaporation protocol of about 1h.

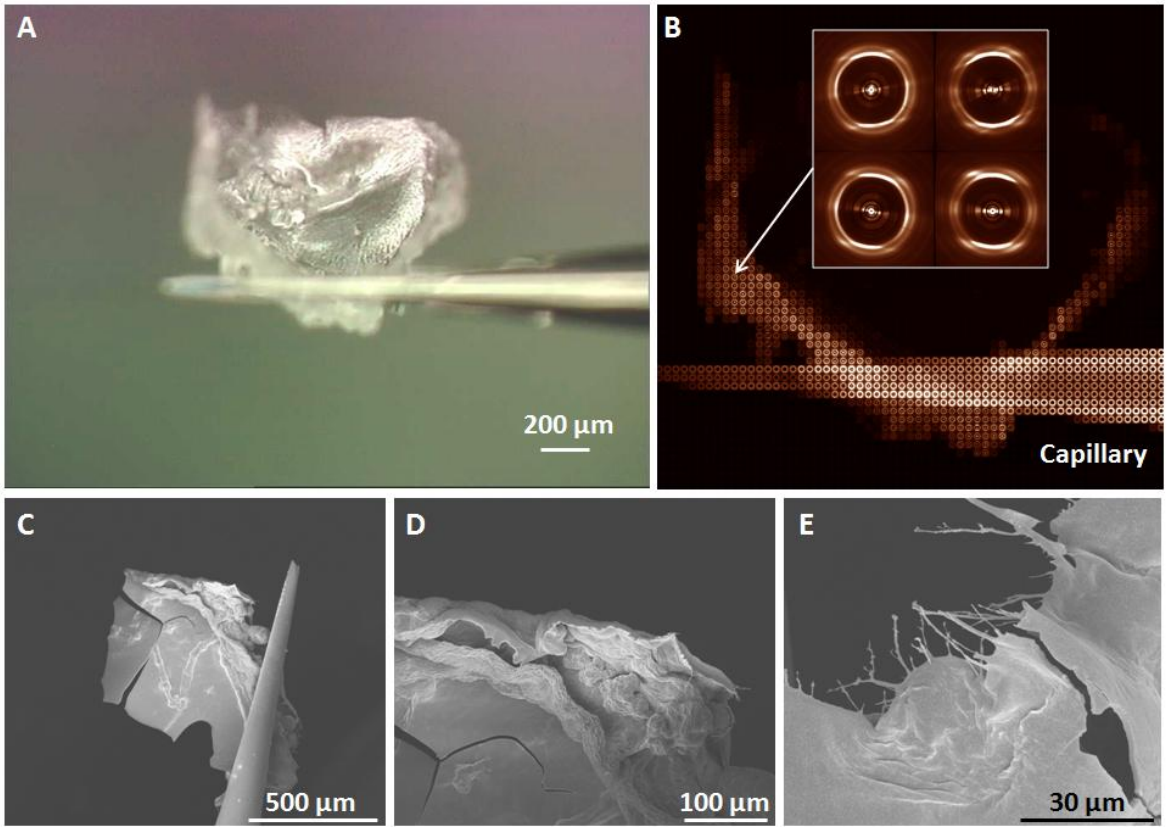

Figure 7 A: Optical image of Aβ(25-35) free-standing residue (attached to a glass capillary tip) dried on a superhydrophobic Si_3N_4 membrane; B: µXRD mesh scan of the residue in figure 7A (inset: close up on residue edge µXRD patterns); C-E: SEM micrographs of the Aβ(25-35) residue in figure 7A.

A particularly well-ordered XRD pattern coming from the outer rim of the free-standing residue reveals an orthogonal cross-β structure with a number of equatorial peaks (Figure 8A). The a-axis is chosen along the meridional direction of the cross-β-peak and the b/c axes along the equatorial direction.[43] The reflection on the meridian with d = 0.473 nm is indexed as (100) and a d = 0.239 nm peak as (200) suggesting parallel β-strands (Table 1).[19,44] The intensity profile of the cross-β peak can be separated into three h0l reflections, the (110) reflection and a

short-range order peak revealing a semicrystalline material (Figure 8B,C). The short-range order scattering (d~0.40 nm) appears to be random. Some of the patterns in the raster-scan show only short-range order scattering due to the absence of a crystalline fraction (not shown). Subtracting such a pattern from a semicrystalline one was used to enhance the visibility of the weak equatorial peaks.

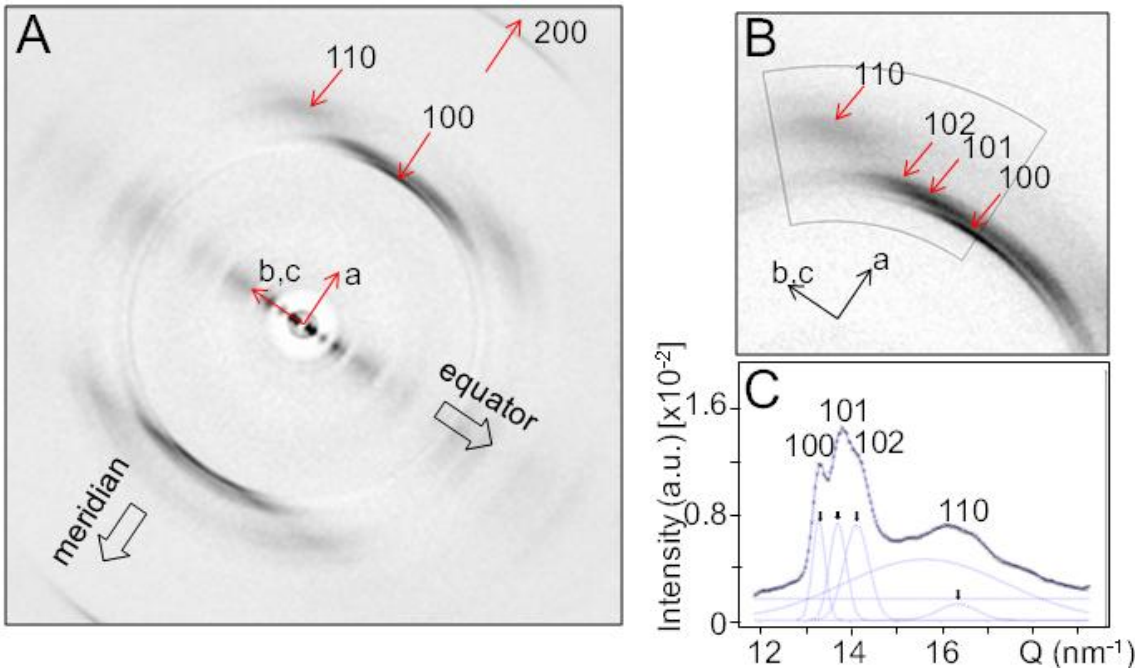

Figure 8 A: XRD pattern the Aβ(25-35) residue from the outer rim of the residue (Figure 7B). The reflections are indexed by an orthogonal cross-β structure. B: Zoom into cross-β-peak revealing a sub-structure due to h0l reflections and the neighboring (110) reflection. C: Radial intensity profile corresponding to the azimuthally integrated sector in (B). 4 Gaussian profiles fitted the h0l and (110) peaks (black arrows), a broad Gaussian for short-range order and a 0-order Lorentzian background. The fitted peaks and the resulting curve are in blue.

The d-spacing list and the deduced unit cell are collected in Table 1.

Table 1 Aβ(25-35) crystallographic parameters based on an orthogonal cross-β structure (The particle size -L- was derived from the radial peak width of fitted Gaussian functions by Scherrer's equation.[49])

hkl	d (nm)	L (nm)	Unit cell parameters (α=β=γ=90°)
(100)	0.473	25	a=0.476 nm
(200)	0.239	14	b=0.658 nm
(101)	0.458	16	c=1.833 nm
(102)	0.445	13	
(110)	0.384	7	

The b-axis of ~0.66 nm is shorter than expected for antiparallel β-sheets (~0.70 nm) supporting parallel β-sheets. The equatorial peaks extending to low-angles suggest scattering due to amyloidal nanofilaments which has been studied extensively for other self-assembled oligopeptides and Aβ-fragments.[45-47]

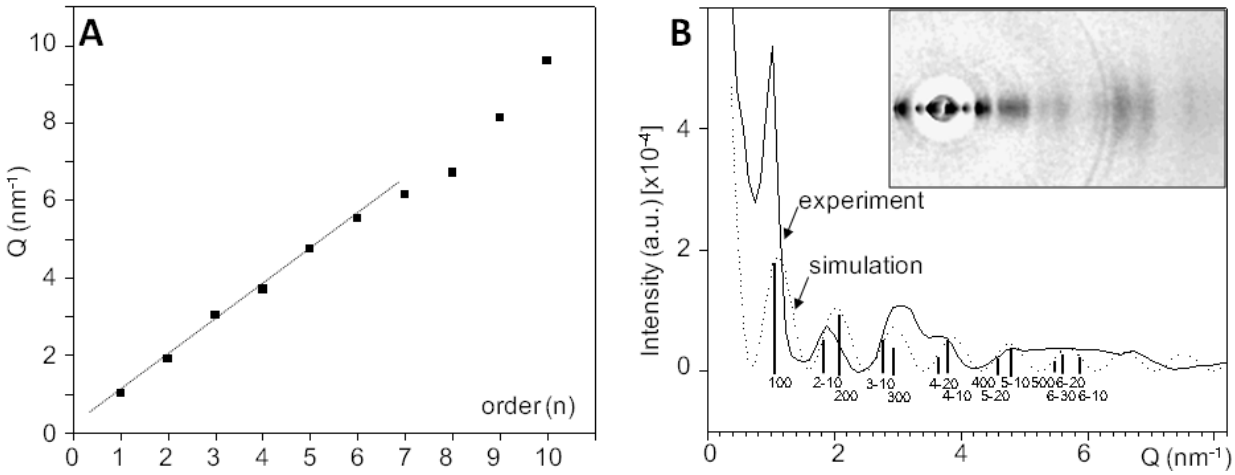

Figure 9 A: Plot of position of low-angle orders against the order number. A linear regression curve with a slope of 0.91 (σ=2) nm^{-1} has been fitted to the first 6 orders. B: Experimental intensity profile of equatorial scattering (solid line) derived by azimuthal integration across the peaks shown in the inset. Random short-range order scattering at the position of the β-peak has been subtracted in order to enhance the visibility of the peaks. The simulated scattering of a tube is shown as dotted line. The vertical bars correspond to a hexagonal lattice of close-packed tubes.[45] Only one of each set of symmetry-related reflections is shown.

The positions of the individual peaks were determined by fitting Gaussian profiles (not shown). The orders n=1 to 6 show a linear Q-dependence with a slope of 0.91(σ=2) nm^{-1} (Figure 9A). For orders n≥7 we observe a deviation from a linear dependence which is probably be due to 0kl equatorial reflections from the cross-β structure (Figure 9A).

The equatorial intensity trace can be modeled by a cylinder with the 6.9 (σ=1) nm diameter derived from the slope of the order plot (Figure 9; Supporting Information).[45,47] Other models based on solid, double-walled cylinders or β-sheet slabs did not match the cylinder diameter or provided less good fits.[46] The significant difference of the calculated and observed intensities at the position of the 1st order is attributed to a coincidence of the first order interference peak of a hexagonal lattice of close-packed cylinders[48] with the form factor of the cylinder (Figure 9B).

The decrease of the apparent particle size (L), derived from the peak broadenings by Scherrer's equation[49], of L=38 nm for the (100) reflection descending to 5-10 nm for the higher order reflections supports a loss of coherence for the higher-order terms.

A less ordered equatorial trace is observed for several weaker patterns in the inner part of the residue corresponding to a period of about 4.3 nm. The peaks show a larger azimuthal spread and the larger peak width suggests smaller domains than for the more ordered equatorial trace. A possible origin of this structural feature could be a partial melting of β-sheet strands as predicted by molecular dynamics (MD) simulations.[50] The comparable weaker equatorial features suggest, however, that this could only affect a small volume fraction while extensive loss of β-sheet structure occurs a 309 K according to MD simulations.[50] It might therefore be interesting to probe the thermal behavior of amyloid residues by combined nanocalorimetry and nanodiffraction[51] in the future.

Conclusions

In this work we showed how the presence of superhydrophobic surfaces can sensibly influence the aggregation mechanisms of Aβ peptides involved in the formation of fibroidal plaques at the origin of Alzheimer's disease.[3,15,16] In order to determine the structural configuration of the peptides under investigation by synchrotron μXRD techniques, we fabricated novel X-ray transparent superhydrophobic Si_3N_4 membranes. Our findings show how the comparison between the solid residues consisting of several Aβ peptides, namely Aβ(1-40), Aβ(12-28) and Aβ(25-35), highlights peculiar conformational differences in presence of a hydrophilic or superhydrophobic support. We observe a general enhancement of the fibril self-assembly processes due to recirculating microfluidic flows and homogeneous evaporation rate provided by superhydrorepellent surfaces.[17,52] The quasi contact-free nature of the drying droplets allows the

formation of free-standing residues, which are homogeneously characterized by an extended fibrillar structure if compared with the ring-like substrate-adherent moieties obtained in hydrophilic conditions. Aβ(25-35) is particularly predisposed to form fiber textured structures which resulted quasi-crystalline over the SH Si_3N_4 membrane. The protocol that we used is a simple and straightforward method to monitor structural changes that can happen in amyloidal proteins. For this reason we envisage the extension of such study to biocompounds involved in other neurodegenerative diseases (*e.g.* α-synuclein/Parkinson's,[4] huntingtin/Huntington's[5]) and non-neurodegenerative ones (*e.g.* amylin/diabetes mellitus type II[6,7]). Further, the use of micro- and nano-structured supports to manipulate amyloidal soft matter could be also exploited as a smart fabrication protocol for the rapid realization of amyloidal fibers, which find application in tissue engineering, cell adhesion[29,30,34,53] and other materials applications.[54-56]

ASSOCIATED CONTENT

Supporting Information Available: Additional information on the simulation of equatorial X-ray scattering, the experimental setup and the membranes related contact angles. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Dr. Angelo Accardo, Istituto Italiano di Tecnologia, angelo.accardo@iit.it, +3901071781847.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

SHS, superhydrophobic surface; Aβ, Amyloid beta; TFA, trifluoro-acetic acid; μXRD, X-ray micro-diffraction; SEM, Scanning Electron Microscopy; CA, contact angle; AD, Alzheimer's disease; PMMA, poly(methyl methacrylate); MD, molecular dynamics.

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