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Detection of Amyloid- β Fibrils Using Track-Etched Nanopores: Effect of Geometry and Crowding

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20 Several neurodegenerative diseases have been linked to proteins or peptides that are prone
21 to aggregate in different brain regions. Aggregation of amyloid- β ($A\beta$) peptides are
22 recognized as the main cause of AD progression, leading to the formation of toxic $A\beta$
23 oligomers and amyloid fibrils. The molecular mechanism of $A\beta$ aggregation is complex
24 and still not fully understood. Nanopore technology provides a new way to obtain kinetic
25 and morphological aspects of $A\beta$ aggregation at single-molecule scale without labelling,
26 by detecting the electrochemical signal of the peptides when they pass through the hole.
27 Here, we investigate the influence of nanoscale geometry (conical and bullet-like shape) of
28 a track-etched nanopore pore as well as the effect of molecular crowding (polyethylene
29 glycol-functionalized pores) on $A\beta$ fibril sensing and analysis. Various $A\beta$ fibril samples
30 that differed by their length were produced by sonication of fibrils obtained in the presence
31 of epigallocatechin gallate (EGCG). The conical nanopore functionalized with PEG 5 kDa
32 is suitable to discriminate of the fibril size from *relative current blockade*. The bullet-like
33 shape nanopore enhances the amplitude of the current and increases the dwell time
34 allowing to well discern the fibrils. Finally, the nanopore crowded with PEG 20 kDa
35 enhance the relative current blockade and increase the dwell time, however, the
36 discrimination is not improved compared to the “bullet-shape” nanopore.

37

38

39 1. Introduction

40 One central event in Alzheimer's disease (AD) is the formation of cerebral senile plaques
41 consisting of aggregated amyloid- β (A β) peptides¹⁻³. Their mechanism of aggregation is
42 currently not fully understood due to the coexistence of numerous transient species at each
43 stage of aggregation⁴. However, the A β peptide assembly occurs through three main steps
44 that can be characterized by the intermediates that compose the reaction⁵. The first step
45 corresponds to a lag phase that contains small amyloid intermediates (oligomers). Then,
46 the second step leads to the formation of mature oligomers allowing a rapid exponential
47 growth of their size. During this, the A β intermediates are highly polydispersed and small
48 oligomers and fibrils coexist. After reaching the plateau phase of aggregation, fibrils
49 undergo a slow maturation over time. At this step, small intermediates are almost no longer
50 present and morphological changes in fibrils can be tertiary or quaternary in origin⁶. To
51 fully understand the dynamic of the A β aggregation process, it is required to obtain the
52 information about the kinetic and the morphology of the aggregate that is impossible
53 presently to obtain with a single method. Therefore, an experimental technique is needed
54 to study both aspects. The development of such technique is important to improve our
55 knowledge of amyloid aggregation but also the treatment and the early diagnosis of AD.

56 The nanopore technology can detect objects at the single molecule level⁷. The classical
57 experiments consist in recording the current perturbation during the translocation of an
58 object under an applied voltage bias⁸. These perturbations give information about the
59 properties of the object including volume, shape or sequence⁹⁻¹¹. The first demonstration
60 of the A β peptide detection by nanopore was done using α -hemolysin¹². The latter allow
61 discriminating the random coil vs β -sheet structure of A β 25-35, based on the amplitude of
62 the relative current blockade¹³. However, the main limitation of the α -hemolysin is the

63 small aperture where the A β fibrils (or large oligomers) cannot pass through generating
64 only bumping events¹⁴. Compared to biological nanopores, the geometry of the solid-state
65 ones can easily be tuned from a couple of nm to μ m diameter with high or low aspect ratio
66 (defined from the ratio length/diameter)¹⁵. This allows the detection of various
67 biomacromolecules from folded to unfolded proteins to virus¹⁶⁻²⁰. Thanks to an adjustable
68 the pore size, the detection of protein aggregates by solid-state nanopore is currently an
69 active field²¹. Silicium Nitride (SiN) nanopores allow the discrimination of amorphous and
70 ordered aggregates as well as small oligonucleotide^{9,22,23}. However, intrinsically disordered
71 proteins require a surface functionalization of the silicon nitride to prevent the nanopore
72 fouling¹⁰. This can be achieved by different coating of the nanopore surface by
73 phospholipids, surfactants or PEG grafting^{22,24,25}. On the other hand, the fibril detection is
74 limited by the size of the nanopore aperture as illustrated by the bumping events reported
75 for the A β aggregates detected by nanopipette^{14,26}. To overcome this limitation, we
76 proposed to use a conical nanopore obtained by the track-etched method in polymer film
77 functionalized by PEG to prevent the fouling²⁷. Using this strategy, β -lactoglobulin and
78 Tau amyloids were detected, by their passage from the large to small aperture²⁸. A similar
79 strategy was also reported to detect α -synuclein aggregates using a nanopipette²⁹. Despite
80 a low resolution, the polymer track-etched nanopore presents numerous advantages. First,
81 the intrinsic method of fabrication allows the nanopore being already filled with an aqueous
82 solution after chemical etching, limiting thus the problem of the wettability. In addition,
83 the surface modification is easy to achieve in aqueous solution and it is possible to know
84 the surface density of the grafting³⁰. Finally, the track-etched nanopores are exceptionally
85 stable after PEG grafting and can be used for resistive pulse experiments for several

86 weeks^{28,31}. This was also the case for the SiN functionalized with PEG incorporated in
87 microfluidic chamber³².

88 Despite the feasibility of investigating protein aggregation, the nanopore technology
89 shows limitations to study the A β fibrils due to their adsorption and thus such goal is still
90 challenging. In addition, all the previous studies were done on heterogenous samples and
91 thus the real morphology and volume of amyloid are not controlled. This work aims to
92 overcome this limitation by proposing an optimal nanopore to detect well-calibrated A β 42
93 fibrils. Based on our previous studies^{28,33}, we have considered the conical track-etched
94 nanopore as the most suitable to detect small fibrils thank to the combination of a large
95 aperture favouring fibre trapping and the small one as sensing zone²⁸.

96 To demonstrate that, we have designed, a conical track-etched nanopores and to detect
97 well-calibrated small fibrils of A β 42 peptides. Then, we also ask the question about the
98 optimal conditions to discriminate different sizes of fibrils in terms of pore geometry. We
99 have considered two potential strategies to improve the fibril discrimination: the nanopore
100 geometry and the crowding. Concerning the geometry the “bullet-like” shape nanopore
101 allow reducing the detection zone compared to the conical shape nanopore. We hypothesize
102 that this will enhance the amplitude of the current blockade and thus expect an
103 improvement of the discrimination of fibre size. On the other hand, several previous
104 investigations obtained using crowded nanopores are consistent with an essential role of
105 auxiliary macromolecules to improve nanopore detection capabilities^{29,34}. According to
106 that, here we have considered crowding the tip aperture of the nanopore. In this strategy,
107 we hypothesize that the crowding will increase the dwell time of fibre inside the nanopore

108 but also amplitude of the current blockade. Thus, we could also expect to better
109 discriminate the fibril with different sizes than conical nanopore.

110 To verify our two hypotheses and finally propose a suitable nanopore geometry to detect
111 and discriminated A β 42 fibrils fibril, we designed track-etched nanopores, from conical to
112 bullet-like shapes. The nanopore were functionalized with PEG 5 kDa and 20 kDa to induce
113 a crowded environment. In parallel, we produced and characterize by microscopy well-
114 calibrated A β 42 fibrils with different sizes. Then, we detect of calibrated A β 42 fibres using
115 the different nanopores (conical, “bullet-like” shape and crowded). For each nanopore, the
116 experimental result was discussed in order to compare the different strategy. Finally,
117 molecular dynamics simulations have been also conducted to understand at the nanoscale
118 the translocation of the fibrils in a crowded nanopore.

119 2. Materials and methods

120 2.1. Nanopore design

121 Single polymer nanopore was obtained by the track-etched method. The single tracks
122 were produced by Xe irradiation (8.98 MeV u⁻¹) (GANIL, SME line, Caen, France) of
123 polyethylene terephthalate (PET) film (thickness 13 μ m or 6 μ m, biaxial orientation,
124 Goodfellow).

125 The conical nanopores were obtained as previously reported^{35,36}. First, the tracks were
126 activated by UV exposition 12 h per side (Fisherbioblock; VL215.MC, λ =312 nm). Then,
127 the chemical etching was performed under unsymmetrical condition (etchant solution 9 M
128 NaOH and stop solution 1 M KCl and 1 M of acetic acid) using the electrostopping method
129 (1 V). The opening of the pores was monitored using an e-one amplifier or EPC 10 Heka
130 amplifier (Element, Italy and Heka electronic, Germany). After nanopore opening, the tip

131 radius r was deduced from the conductance G (measured from -100 mV to 100 mV) with
132 NaCl solution at different concentrations, assuming bulk-like ionic conductivity inside the
133 nanopores using Equation (1).

$$134 \quad G = \frac{\kappa\pi rR}{L} \quad (1)$$

135 where κ is the conductivity of the solution, L the nanopore length and R the radius of the
136 base side. The latter is obtained from the SEM image performed on multipore membranes
137 opened under strictly the same conditions (see Figure SI-2).

138 The single bullet shape nanopore was obtained following the method previously
139 reported^{37,38}. Briefly, only one side of the tracked film is activated by UV for 26 h
140 (Fisherbioblock; VL215.MC, $\lambda=312$ nm). Then, the chemical etching was performed under
141 symmetrical conditions with NaOH 6M and Dowfax2A1(EZkem, USA) 0.05% v/v at 60°C
142 for 8 min. At the end of the etching process, the nanopore was washed in several pure water
143 (18.2 MOhm) baths.

144 The scanning electron microscope (SEM) images were performed on multipore
145 membranes (10^8 pores/cm²), using a Thermo Scientific Quattro ESEM, at high vacuum at
146 10 kV and 46 pA or Hitachi S-4800.

147 2.2. Nanopore functionalization

148 The pores were functionalized with polyethylene glycol amine (PEG-NH₂) to reduce the
149 fouling or to crowd the sensing zone. To do so, the nanopores were immersed overnight in
150 a solution of PEG-NH₂ 5kD for pores 1 & 2 (or PEG-NH₂ 20kD for pore 3), MES 0.1 M
151 and EDC 50 mM in KCl 0.1M. The success of the functionalization was evidenced by
152 current-voltage measurements (See Figure SI-1).

153 2.3. Production of A β 42 fibrils

154 2.3.1. A β 42 fibril assembly

155 Synthetic human A β 42 peptides were purchased from the ERI Amyloid Laboratory
156 LLC. A β peptides were dissolved at a concentration of 8.5 mg mL⁻¹ in a solution of
157 guanidine thiocyanate (Sigma-Aldrich) at 6.8 M. Then, the solution was sonicated for 5
158 min at 52°C and ultrapure water was added to reach a final concentration of 5 mg mL⁻¹ of
159 A β peptides and 4 M of guanidine thiocyanate. The solution was centrifuged at 10000×g
160 for 6 min at 4°C and the supernatant was collected and filtered (polyvinylidene fluoride
161 (PVDF); 0.45 μ m). Then, the supernatant was injected into a Superdex® 75 Increase
162 10/300 GL column (GE Healthcare Life Science) equilibrated with a solution of 10 mM
163 sodium phosphate buffer (pH 7.4). A flow of 0.5 mL min⁻¹ was used to collect the peak
164 corresponding to monomeric A β 42. After the purification, the concentration of peptides
165 was determined with a NanoDrop™ 8,000 spectrophotometer (Thermo Scientific). Finally,
166 monomeric A β 42 peptides were aliquoted and stored at -80°C until using.

167 The A β 42 fibrils were obtained as follow. The stock solution was diluted to reach 30 μ M
168 and were exposed to 25 μ M of EGCG or the vehicle alone (0.05% DMSO, v=v) in a 10 mM
169 sodium phosphate buffer at pH 7.4. Peptide solutions, final volume of 600 μ L, were let to
170 aggregate in low-binding Eppendorf with 30 μ M Epigallocatechin Gallate (EGCG) (1:1)
171 or the vehicle alone (1% dimethyl sulfoxide, DMSO). Tubes were arranged vertically at
172 25°C without shaking (quiescent state). Thioflavin T (ThT) binding assay was used to
173 monitor the fibril formation. 20 μ l of A β 42 solution aliquots were withdrawn and mixed
174 with 14 μ l of glycine-sodium hydroxide buffer (142 mM at pH 8.3) and 6 μ l of ThT at 100
175 μ M. The mix was placed into a 96-well plate of black polystyrene with clear bottoms and
176 a polyethylene glycol coating (Thermofisher Scientific). The fibrils formation was

177 monitored by following fluorescence of ThT ($\lambda_{\text{ex}}=445$ nm and $\lambda_{\text{em}}=485$ nm) with a
178 Fluoroskan Ascent™ microplate fluorimeter (Thermofisher Scientific). Aggregation
179 curves were generated with Matlab software (Matlab R2020a), and each kinetic trace was
180 fitted independently using the curve fitting tools.

181 2.3.2. Fragmentation of A β 42 fibrils

182 The fragmentation of the A β 42 fibrils harvested at the plateau phase (see fig 1A and
183 figSI-5) were performed at a concentration of 15 μ M in 10 mM PBS by sonication at
184 different energies either using a wet bench sonicator 34 W (Branson Ultrasonic model 200)
185 for different times from 5 min to 45 min or a cup-horn sonicator with a power of 300 W in
186 presence of two beads (to enhance sonication process) for 15 s or 60 s. After sonication,
187 the samples were frozen and stocked at -80°C.

188 2.3.3. Transmission electron microscopy (TEM) - characterization

189 Samples of A β 42 fibrils were deposited onto Formvar carbon-coated grids, negatively
190 stained with freshly filtered 2% uranyl acetate, dried. The TEM images were performed
191 using a JEOL 1400 electron microscope at an accelerating voltage of 80 kV. The
192 measurements of the fibrils length and diameter were performed using ImageJ software
193 and analysed with matlab software.

194 2.3.4. A β 42 Fibers detection using nanopore

195 The A β 42 fibrils were detected using the resistive pulse method. To do so, the single
196 nanopore was mounted between two Teflon chambers containing electrolyte solutions.
197 PBS 1X at pH 7.2 or NaCl 1M PBS, pH 7.2 was used. Electrical measurement was
198 performed using a patch-clamp amplifier (EPC10 HEKA electronics, Lambrecht,
199 Germany). The current was measured by electrode Ag/AgCl. For all the experiments, the

200 ground was placed in the chamber containing the A β 42 fibrils (concentration 50 nM or 100
201 nM monomers). A positive voltage is applied on the working electrode, placed to the
202 opposite reservoir. The data was acquired with a frequency rate of 20 kHz or 40 kHz or
203 100 kHz. A Bessel filter of 10 kHz was used during the recording. The data analysis was
204 performed using a custom-made LabView software PeakNano Tools. Briefly, a Savitzky–
205 Golay filter order 1 was used to correct the base line fluctuations. The detection of events
206 was performed using a threshold of 4σ (σ being the standard deviation to the baseline
207 signal). Each event was characterized by the relative current blockade ($\Delta I/I_0$), the dwell
208 time (Δt), the slopes both at the beginning and the end of the blockade. The parameters of
209 the current blockade were then analysed using Matlab software with custom scripts.
210 Statistical comparisons of the Δt and $\Delta I/I_0$ were performed using t-test or welch-test for the
211 samples with no equal variance. A probability level (p value) of 0.05 was chosen for
212 statistical significance.

213 2.4. Molecular dynamics simulations

214 To model the resistive pulse measurements, we used classical all-atom MD simulations
215 in the NAMD.2.12 package framework³⁹ with CHARMM36 force-field parameters⁴⁰. The
216 water (described with the TIP3P model) box was chosen as large enough to prevent any
217 interaction between each periodic cell. KCl ions (at a concentration of 1 M) were used to
218 reproduce the experimental environment. We worked during the simulations in the NPT
219 ensemble where temperature (300K via Langevin dynamics method⁴¹) and pressure (1 atm
220 via Langevin piston method⁴²) were kept constant. To take into account the long-range
221 electrostatic forces the classical particle mesh Ewald (PME) method⁴³ with a grid spacing

222 of 1.2 Å, and a fourth-order spline interpolation was used. The integration time step was
223 equal to 1 fs.

224 2.4.1. Description of the conical nanopore.

225 Our conical nanopore was described in previous papers^{30,44}. Its dimension ($R_{tip} = 3$ nm,
226 $R_{base} = 3.5$ nm, length=14.8 nm) was obtained through the association of several carbon
227 nanotube sections of different radii. The chemical structure of PET nanopore as used in the
228 experiments was modelled by replacing one third of the carbon atoms constituting the
229 nanopore by oxygen atoms. Then partial charges were imposed to carbon (positive) and
230 oxygen (negative) atoms while keeping global neutrality of the nanopore. According to the
231 experimental functionalization 30 hydroxyl functions (0.10 M) were then randomly
232 functionalized to carbon atoms and three (twenty-four) PEG 5 kDa (3 kDa) were added as
233 in the experiments to model the conical (bullet shape) nanopore. Finally, the functionalized
234 nanopore was solvated and attached to 2 solvated and ionized reservoirs (dimensions equal
235 to $133*133*68$ Å³). The complete system dimensions are $133*133*285$ Å³, for a total of
236 around 350.000 atoms. The optimization of the systems followed three successive
237 procedures. First, we minimized the energy of the total system at 0 K. Then, the system
238 was progressively thermalized to a temperature equal to 300 K. Finally, the system evolved
239 in the NPT ensemble, and physical observables were calculated using time averages.
240 During all simulations, every atom constituting the conical nanopore wall was kept fixed.
241 This system was relaxed for 100 ns, allowing for PEG to relax around the surface of the
242 nanopore. To determine the ionic currents, various electric fields were applied using
243 simulations in the NVT ensemble. The determination of the ionic current values was
244 performed according to the procedure described previously.

245 2.4.2. Description of the simulated amyloids.

246 We studied the behaviour of two different structures: 2NAO [50] (~2000 atoms) and
247 5OQV⁴⁵ (~3000 atoms), two dual subunit amyloid fibril structures translocating through
248 the two previously described nanopores. Our objective is to study the role of PEG density
249 (crowding) on the current perturbation inside the nanopore. The translocation of these big
250 amyloid structures (both close to the tip dimensions) will allow us to understand the
251 dynamic of PEG during such protein displacement in a crowded environment. For each
252 simulation run, the already relaxed protein (for at least 5 ns) was placed at the entrance of
253 each functionalized nanopores and let diffusing inside the conical nanopore under an
254 electric field equal to 2 V.

255 3. Results and discussion.

256 3.1. Nanopore and amyloid characterization

257 3.1.1. Nanopore characterization

258 Two methods, electrostopping and surfactant controlled, were used to produce
259 unsymmetrical nanopore, leading to conical and bullet-shaped pores respectively.
260 Electrostoping was used to obtain conical-shape nanopore. The size of the nanopore was
261 determined from the conductance measurement and SEM analysis (Figures SI-1 & SI-2).
262 After etching, the nanopores were coated with PEG to prevent current rectification and
263 protein adsorption^{22,28}. Two conical nanopores were obtained. The first one (noted pore 1,
264 $d=40\pm 2.5$ nm, $D=540\pm 90$ nm, and 6 μm thick) was functionalized with PEG 5 kDa (Table
265 SI-1). The other one (noted pore 3, $d=28\pm 2$ nm $D=635\pm 115$ nm with a thickness of 13
266 μm) was functionalized with PEG 20 kDa (Table 1 in SI). The success of the nanopore
267 functionalization is directly characterized by a decrease of the current rectification as well

268 as the nanopore conductance due to the grafting on carboxylate ending moieties by
269 uncharged PEG chains at the surface, as previously demonstrated³⁰(Figure SI-1).

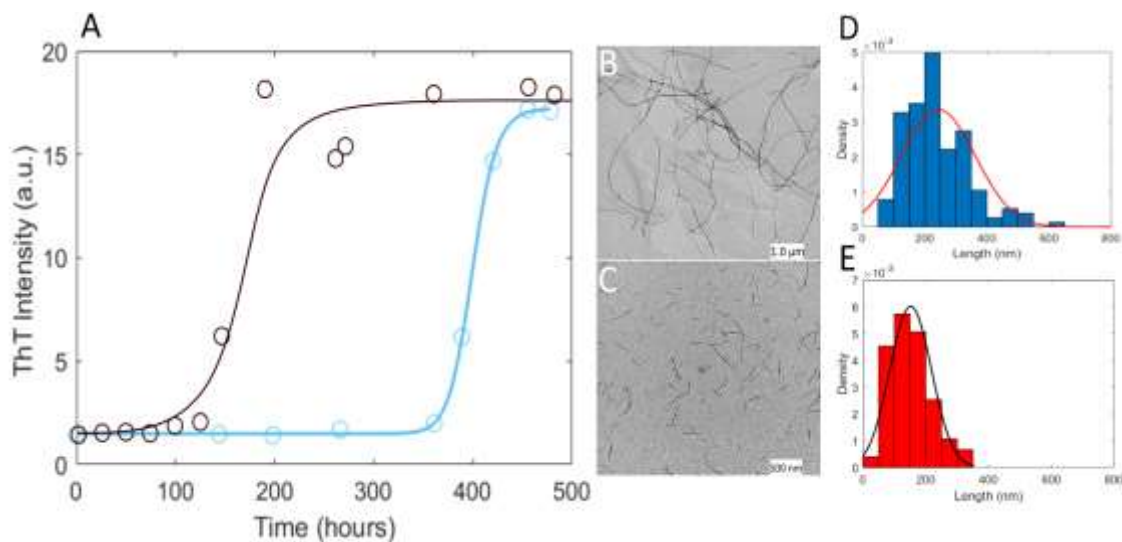
270 The bullet-like shape nanopore was obtained using surfactants. The current-voltage
271 dependences of the nanopore before and after PEG are reported on the SI-1. After the
272 etching the pore exhibits a base diameter of 379 ± 9 nm and a tip diameter of 78 ± 4 nm)
273 (noted pore 2). The functionalization is characterized by a decrease of the conductance at
274 NaCl 0.5M and the loss of ionic current rectification (Figure SI-1b).

275 3.1.2. A β 42 Fibrils characterization

276 To validate the fibrils detection capacities of our nanopore sensing, we have to obtain
277 well-defined A β 42 fibrils with different sizes as standard. To produce such fibrils, we first
278 aggregated A β 42 monomers until reaching a plateau (black curve figure 1A) and then
279 sonicate the fibrils. Contrary to our expectations, the sonication did not allow the
280 fragmentation of the fibrils (Figure SI-3). It is well known that numerous molecules can
281 interact with A β 42 peptides and therefore modulate their assembly in terms of kinetic and
282 structure. Among them, EGCG is a polyphenol that slow down the aggregation (Figure
283 1A). Indeed, without EGCG, the plateau of the A β 42 aggregation is reached after 200 hours
284 while in presence of EGCG, it is reached after more than 400 hours. The TEM image shows
285 that in presence of the EGCG, the fibrils are quite long (up to several microns, figure 1B)
286 and are not self-associated in large clumps as observed for fibrils formed by pure A β 42
287 peptides (Fig SI-3). We hypothesized that this structure could be related to a greater
288 fragility compared to A β 42 fibrils without EGCG. Therefore, EGCG could make the fibril
289 breakable by sonication. The fibrils formed in presence of EGCG harvested at the plateau
290 phase were sonicated using a classical wet bench sonicator (34 W) for 5 and 45 min. After

291 sonication, fibrils exhibit the rod-like shape (Figures 1C and SI-4) with a diameter centred
 292 at about 20 nm regardless the sonication time. The length of the fibrils is found to be centred
 293 at 247 nm, and 151 nm for the sample sonicated for 5 and 45 min respectively (Figures 1D,
 294 E and SI-4). We notice few aggregates composed by fibrils especially for the sample
 295 sonicated 5 min.

296



297

298 Figure 1 A) Kinetic of A β 42 assembly in absence (black) or in presence of EGCG (cyan).
 299 TEM images of the fibrils containing EGCG harvested in plateau phase (cyan curve) before
 300 B) and after C) 45 min of sonication by wet bench sonicator. Distribution of the fibrils
 301 length sonicated by wet bench sonicator D) 5 min (centred value of length = 247 nm, n =
 302 153 – see also SI-4) and E) 45 min by wet bench sonicator (centred value of length = 151
 303 nm, n = 150).

304 3.2. Fibrils detection using conical nanopore

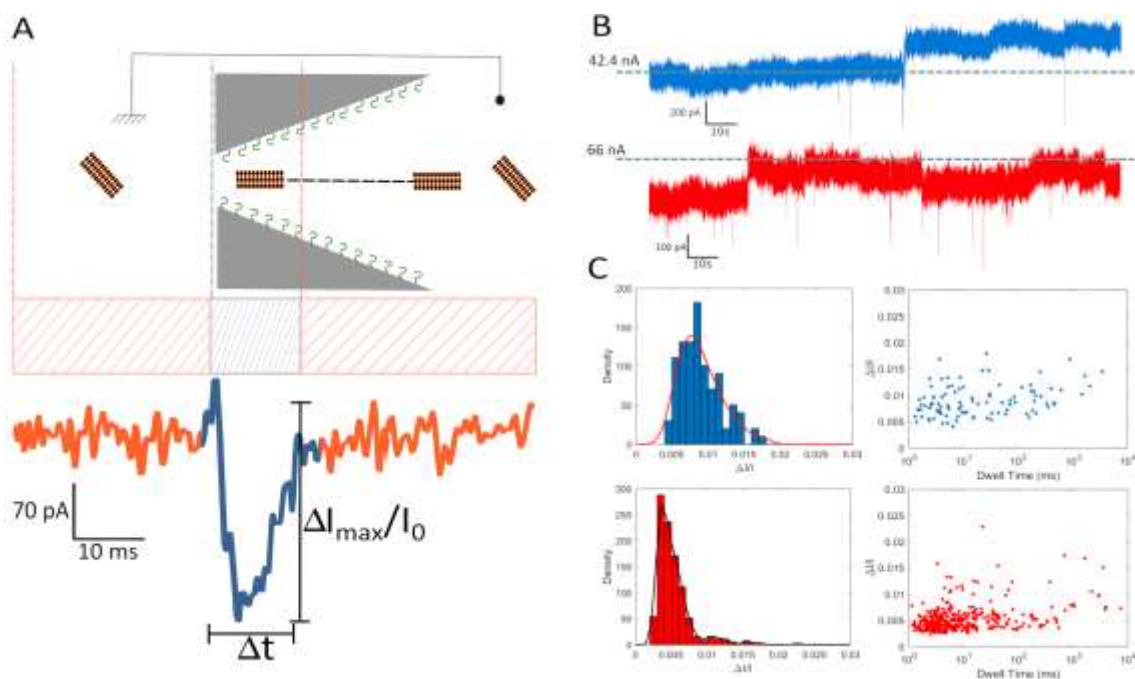
305 We detected the sonicated fibrils using the conical nanopore ($d = 36 \pm 2.5$ nm and $D = 540$
 306 ± 90 nm) coated with PEG 5 kDa to reduce the fouling from A β fibrils. Under this coating
 307 and with respect to the Flory diameter⁴⁴ of PEG under good solvent conditions (5.9 nm),

308 we can assume that the nanopore is not under crowded condition. Usually, the detection of
309 amyloid is performed from the base to the tip side of the nanopore to decrease the energy
310 barrier of entrance as previously reported for β -lactoglobulin and tau^{33,46}. According to
311 that, the first set of experiments was performed in this classical method as previously
312 reported. Typically, the A β 42 fibrils were added on the base side to reach a final
313 concentration of 1 μ M (equivalent monomers). A positive bias (2 V) was then applied to
314 the trans-chamber (Figure 2A). Interestingly, trapping events were observed with the
315 sample sonicated 5 min. We observed a continuous current decrease when the sample
316 sonicated 5 min was added (Figure SI-6). Likely due to fouling, it hinders the translocation
317 of amyloid through the pore. To overcome this problem, we attempted to detect the fibrils
318 from the tip to the base side of the nanopore (See figure 2A). In this case, we observed the
319 current blockades as shown in the current traces reported on figure 2B. The event rates
320 were 0.01, and 0.04 events s^{-1} for the sample sonicated 5, and 45 min respectively. This is
321 consistent with the larger fibrils obtained after 5 min of sonication than after 45 min. These
322 large fibrils are thus less concentrated (at equivalent monomer concentration) and have a
323 slower diffusion coefficient. Regardless the sonication time, we observe that the slope of
324 the blockade beginning, corresponding to the fibril entrance is lower but similar (same
325 order of magnitude) that the slope at the blockade ending (Figure SI-7).

326 We report in figure 2C the event maps and distributions of the relative current blockade.
327 As commonly observed for conical nanopores,⁴⁷ the dwell time distribution ranges from
328 0.1 ms to s scale. The scale of the dwell time and the correlation of the capture rate with
329 the sonication time confirm that the current blockade is due to the translocation of the fibrils
330 and not bumping. Interestingly, we can observe that the distribution of the $\Delta I_{max}/I_0$ depends

331 on the sonication time (Figures 2C & SI-8). Indeed, the distributions are centred at 0.008,
 332 and 0.004 for the sample sonicated 5, and 45 min respectively. This result is consistent
 333 with TEM analysis of the fibrils distribution size. In that case, the $\Delta I_{max}/I_0$ depends on the
 334 fibrils length and thus on their volume. The statistical test shows that only the $\Delta I_{max}/I_0$ is
 335 significant (Pvalue <0.05) to discriminate the two samples (Figure SI-8).

336



337

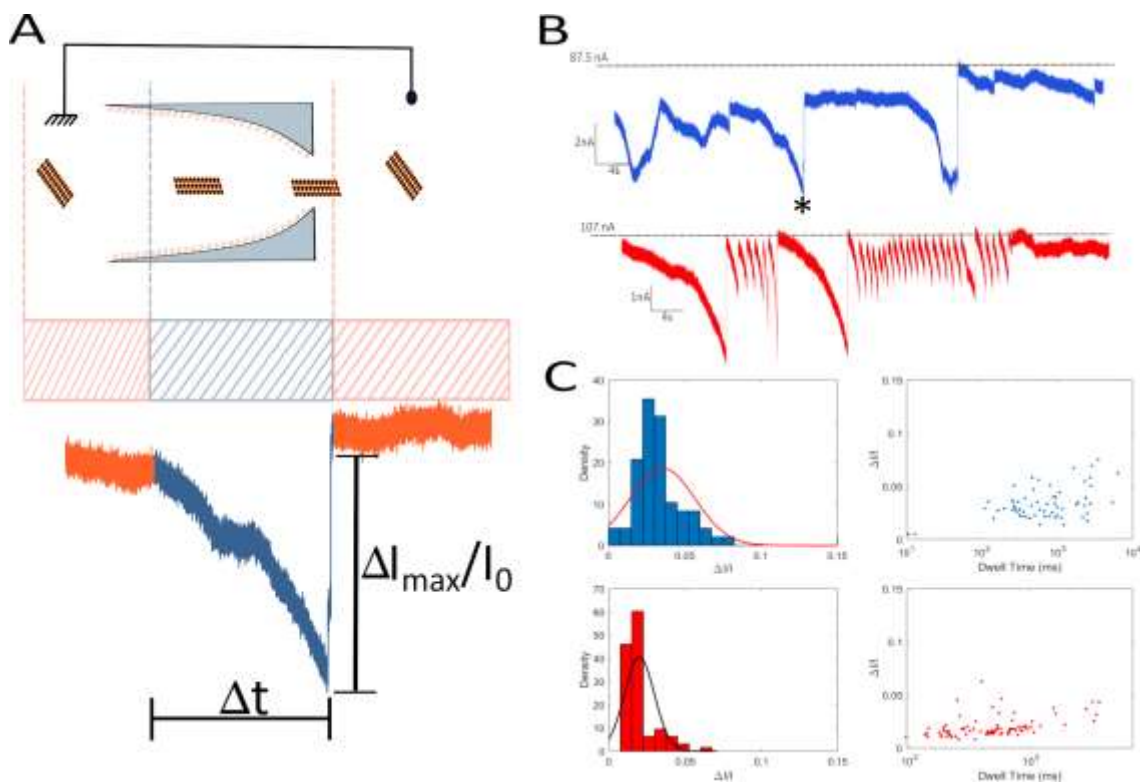
338 Figure 2 A) Top, sketch the fibril translocation in the large conical pore (pore 1) from tip
 339 to base in NaCl 1M (containing PBS 1X, pH 7.2) at 2 V. Bottom, zoom of a current
 340 blockade with different colours representing the fibril outside (orange) and inside (blue)
 341 the sensing zone. The maximum blockade amplitude ($\Delta I_{max}/I_0$) as well the duration of the
 342 event (Δt) are illustrated. B) The current trace recording for the sample sonicated 5 min
 343 (blue) and 45 min (red). C) Top left, distribution of the $\Delta I_{max}/I_0$ of the sample sonicated 5
 344 min (centred $\Delta I_{max}/I_0$ value = 0.008, n = 99). Top right, scatter plot of the $\Delta I_{max}/I_0$ versus
 345 the dwell Time of the sample sonicated 5 min. Bottom left, distribution of $\Delta I_{max}/I_0$ of the

346 sample sonicated 45 min (centred $\Delta I_{\max}/I_0$ value = 0.004, n = 368). Bottom right, scatter
347 plot of the dwell time versus the $\Delta I_{\max}/I_0$ of the sample sonicated 45 min.

348 3.3. Fibril detection using "bullet-like" nanopore

349 We then studied whether "bullet-like" shape nanopores, which present a larger angle in
350 the tip side, improves the resolution of the fibril detection. The dimensions of the "bullet-
351 like" shape nanopore have been calibrated to obtain a tip diameter of about 78 nm and a
352 base of about 379 nm (pore 2) (Figure 3A & SI-1, 2). The pore was also coated with PEG
353 5 kD. The sonicated A β 42 was added to the base side to reach final concentration of 50 nM
354 and 100nM (of monomers) for the sample sonicated 5 min and 45 min respectively
355 (equivalent monomer). A potential bias of 1 V was applied in the trans-chamber. In figure
356 3B are reported examples of the current traces where current blockades can be observed.
357 Firstly, the event rates were found to be 0.08, and 0.25, events s⁻¹ for the sample sonicated
358 5 min and 45 min respectively. This is consistent with the difference of both aggregate size,
359 concentration of fibrils. Contrary to the conical nanopore, we observe a dissymmetry of the
360 blockade event shape. The right side of the blockade, which corresponds to the fibrils exit
361 of the nanopore, has a sharper slope compared to the left one (see figures 3A and SI-9).
362 Both the capture rate and the blockade shape evidence that the events are due to the
363 translocation of the fibrils. In figure 3C, the events map and the distribution of the relative
364 current blockades are plotted. First, we observe that for the A β fibrils sonicated 5 min
365 (centred to 670 ms), the dwell time is significantly longer than the one sonicated 45 min
366 (centred to 482 ms), (Figure SI-10). The centre of the $\Delta I_{\max}/I_0$ is found at 0.030, and 0.017
367 for the sample sonicated 5 and 45 min, respectively (Figures 3C and SI-10). These results
368 are consistent with the TEM and the results from the conical nanopore (pore 1). However,

369 we can notice that the value of $\Delta I_{max}/I_0$ obtained with the bullet-like shape nanopore are
 370 one order of magnitude higher than the one obtained with the conical shape nanopore (pore
 371 1) despite a tip radius twice larger. This can be easily explained by the morphology of the
 372 nanopore. Indeed, the length detection zone of the bullet-shape nanopore is smaller than
 373 for the conical one due to the larger angle. Compared to the conical nanopore, we notice
 374 that both $\Delta I_{max}/I_0$ and dwell time are significantly different to discriminate the two samples
 375 (Figure SI-10). In addition, using the bullet-like shape pore, we observe trapping events
 376 (Figure SI-9) for the sample sonicated 5 min. This was not observed for the sample
 377 sonicated 45 min. A possible explanation could be that after 45 min of sonication time,
 378 there is no residual large size fibrillar bundles in the solution as confirmed by the TEM
 379 analysis.



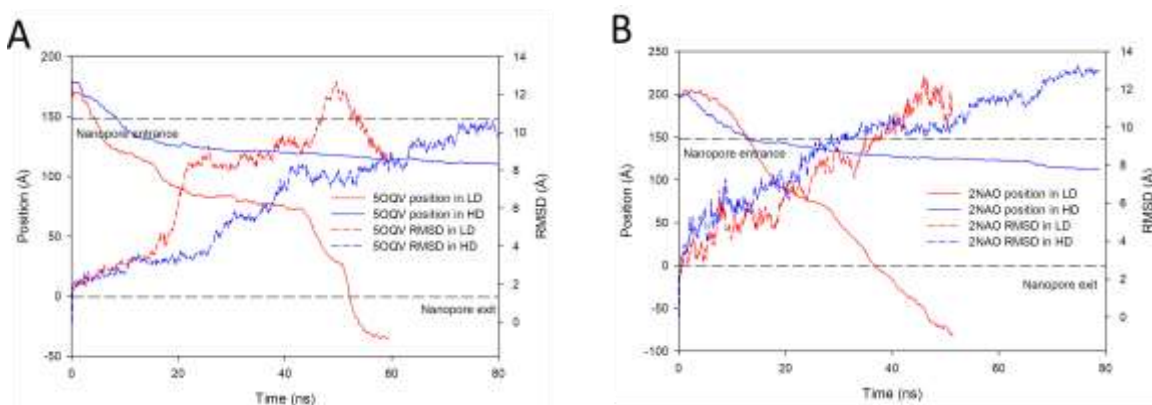
380
 381 Figure 3 A) Top, sketch of A β 42 fibril translocation into a bullet shape nanopore (pore 2)
 382 from base to tip in NaCl 1M (containing PBS 1X, pH 7.2) at 1 V. Bottom, zoom of a current

383 blockade with different colours representing the fibril outside (orange) and inside (blue)
384 the sensing zone. The maximum blockade amplitude ($\Delta I_{max}/I_0$) as well the duration of the
385 event (Δt) are illustrated. B) Current traces recording for the sample sonicated 5 min (blue)
386 and 45 min (red). C) Top left, distribution of the $\Delta I_{max}/I_0$ for the sample sonicated 5 min
387 (0.030, n = 64). Top right, scatter plot representing the $\Delta I_{max}/I_0$ versus the dwell time for
388 the sample sonicated 5 min. Bottom left, distribution of $\Delta I_{max}/I_0$ of the sample sonicated 45
389 min (0.017, n = 84). Bottom right, scatter plot representing the dwell time versus the
390 $\Delta I_{max}/I_0$ of the sample sonicated 45 min.

391 3.4. Fibril detection under crowded nanopore

392 In a recent work, it has been demonstrated that the crowding of one side of the nanopore
393 enhance the detection of α -synuclein fibrils detection ²⁹. On the basis of this previous study
394 and the present results, we have generated a localized crowding by grafting of long PEG
395 chains inside the nanopore. To do so, we have opened a conical shape nanopore (pore 3,
396 $d= 28\pm 1$ nm and $D= 630\pm 110$ nm). After its functionalization with PEG-NH₂ 20 kDa, the
397 measured diameter of the nanopore is about 2 nm, which is in good agreement with the
398 Flory radius in good solvent (13.75 nm) (SI-1, 2 and table 1). Thus, we can reasonably
399 consider that the detection zone is crowded. Using this, we expect to increase the dwell
400 time and the relative current blockade value. To illustrate this assumption, we performed
401 molecular dynamic simulations. Because all-atom simulation does not allow to model such
402 as of the experimental nanopore and A β fibril, we have used a smaller nanopore ($r= 3$ nm,
403 $R=3.5$ nm), functionalized with PEG 5 kDa, and simulated the crowding by tuning PEG
404 density inside the nanopore (Figure SI-11). Two different sizes of amyloid structures were
405 used to understand the influence and role of both nanopores. For both amyloid structures,

406 we observe complete translocations under the 2 V electrical field in the low PEG density
 407 nanopore. The translocation time requires around 60 ns for 5OQV (Figure 4A) and 30 ns
 408 for 2NAO (Figure 4B). For the high PEG density pore, such complete translocations take
 409 much more time and have not been observed within a reasonable simulated time (blue
 410 curves in Figure 4). Nevertheless, in both cases we were able to analyse the behaviour of
 411 the amyloid and more specifically calculate their speed inside the nanopore.
 412



413
 414 Figure 4 : Position of protein centre of mass (full line) and protein RMSD (dotted line)
 415 over time. Low PEG density nanopore simulations are shown in red while high PEG
 416 density nanopore is in blue. The leftmost graph represents 5OQV translocation whereas
 417 rightmost graph shows 2NAO.

418
 419 Plotting the position of the protein over the simulation time shows quite similar
 420 behaviours for the two amyloid structures. We can, however, well distinguish the nanopore
 421 type that have been crossed. In the low PEG density nanopore, we observe two regimes.
 422 The first one depicts free diffusion of the amyloid through the pore while the second one
 423 is characterized by the slowing down of the amyloids that have to interact strongly with

424 the crossed media (nanopore + PEG) to find its way. This behaviour is especially clearer
 425 for large amyloid (5OQV) compared to smaller one (2NAO). In the first regime, the
 426 amyloid is slowing down just after its entering in the pore (~8 ns), then it undergoes heavy
 427 modification of its conformation (as RMSD increases from around 3 to 7.5 Å). At this point
 428 the amyloid starts gaining speed again. Then, from around 20 ns we observe a second
 429 plateau until 45 ns where the RMSD once again abruptly increases, freeing the protein.

430 The amyloids plunged in the high PEG density nanopore also show two distinct, but not
 431 intertwined regimes. Entering the high PEG density nanopore is slightly slower than
 432 entering the low PEG density one as PEG already interacts heavily right at the entrance
 433 with the amyloid. We then observe a shift to a slower but constant speed for the rest of the
 434 simulated time. As the amyloid is now in constant interaction with the functionalization
 435 and the pore, its speed is comparable to the one in the low PEG density nanopore when the
 436 amyloid slows down. Average speeds observed at the different regimes are summarized in
 437 table 1.

438

439

	2NAO		5OQV	
LD nanopore	Time interval (ns)	Speed (m/s)	Time interval (ns)	Speed (m/s)
"Free" movement	3.5-18	0.77	1-6.2	0.84
	27-42	0.69	42.9-55.4	0.86
"Hindered" movement	18-27	0.28	6.2-15.5	0.18
			15.5-22.6	0.29
			22.6-42.9	0.06

HD nanopore				
Pore entrance	1.5-13	0.43	1-11.8	0.39
Inside the pore	13-77	0.05	11.8-100	0.03

440 Table 1: Time analysis and speed values of the two amyloids in the two different conical
441 nanopore (LD for low PEG density and HD for high PEG density functionalization of the
442 conical nanopore).

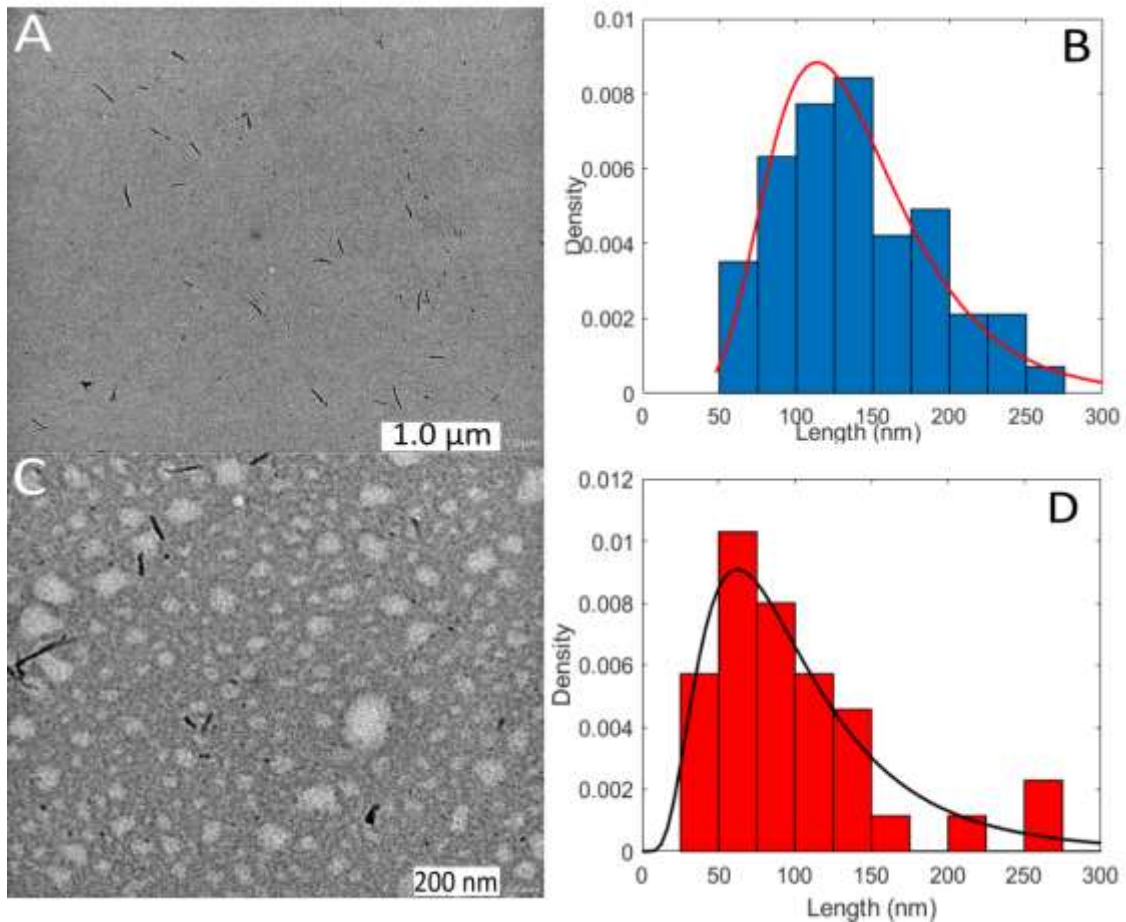
443

444 We also have also reported the current blockades occurring during the translocation (see
445 SI-section Additional result of Molecular dynamic simulation). We have to notice that the
446 MD simulation and the experimental nanopores are too far to make any conclusion about
447 the amplitude of the current blockade. Thus, at this stage, the molecular dynamic
448 simulation only predicts that crowding will allow increasing the amyloid dwell time inside
449 the nanopore due to the PEG/amyloid interactions.

450 3.4.1. Characterization of cup horn fragments

451 Because we use a crowded nanopore, the experiment requires small fibrils to avoid
452 trapping events. To do so, we performed sonication of fibrils assembled in presence of
453 EGCG using a cup horn sonicator (300 W) during 15 s and 60 s (See FigSI-5). As expected,
454 the fibrils are homogeneously broken with a diameter of about 10-15 nm as shown in TEM
455 images (Figure SI-5) and length distributions centred at 114 nm and 61 nm after sonication
456 of 15 s and 60 s respectively (Figures 5B and D).

457



458

459 Figure 5 A) TEM image of the fibrils sonicated 15 s with a cup horn sonicator. B) length
 460 distribution of the fibrils in A (length = 114 nm, n = 57). C) TEM image of the fibrils
 461 sonicated 60 s with a cuphorn sonicator. D) length of the fibrils in C (length = 61 nm, n =
 462 35).

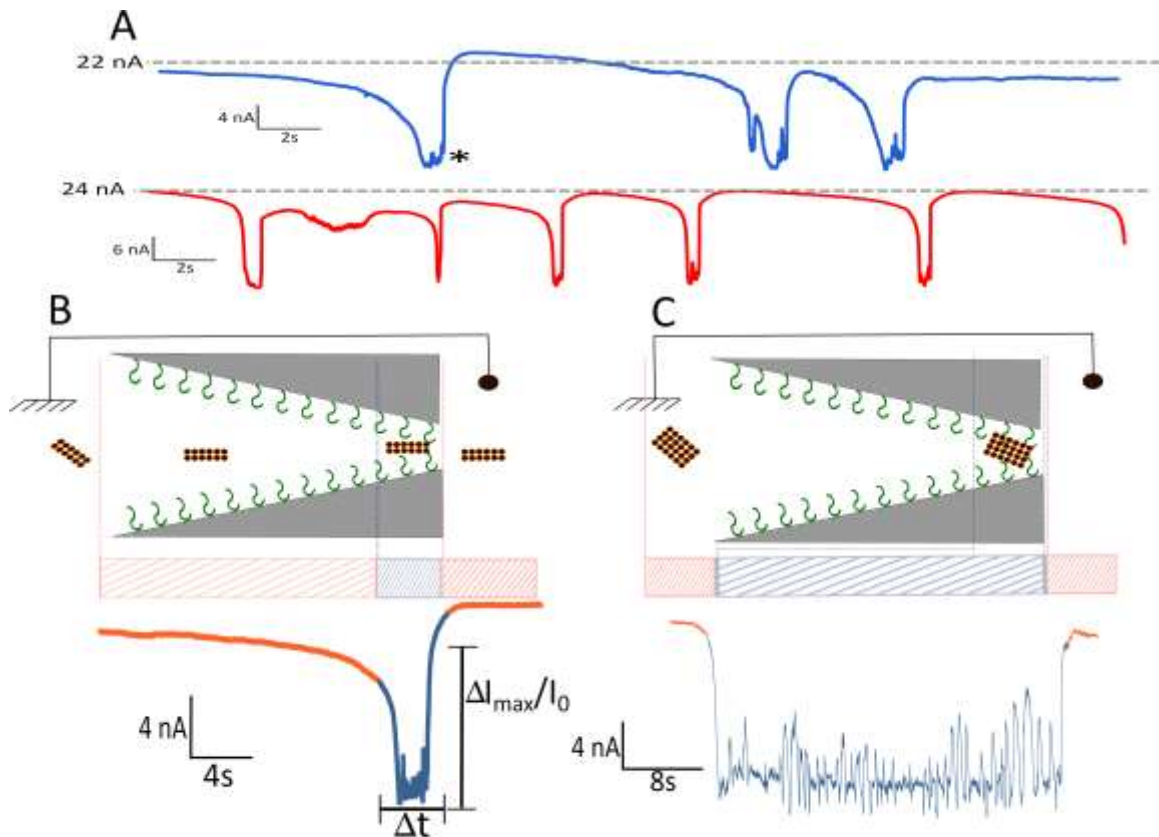
463

3.4.2. Detection of fibrils using conical nanopore with crowded aperture

464

Detection of Aβ42 fibrils was done by resistive pulse experiments. Aβ42 fibrils were
 465 added in the base side (15, 75 or 150 nM) of the pore and a bias of 2 V was applied on the
 466 opposite side. As shown in the figure 6A, Aβ42 fibrils sonicated for 15 s and 60 s were
 467 analysed by recording current blockades.

468



469

470 Figure 6: A) Current trace of translocation events for the samples sonicated 15 s (blue) and
 471 60 s (red) from base to tip in PBS 1X at 1 V. B) Top, sketch of Aβ42 fibril translocation
 472 into a small conical nanopore (pore 3) in crowded condition. Bottom, zoom of a current
 473 blockade with different colours representing the fibril outside (orange) and inside (blue)
 474 the sensing zone. The maximum blockade amplitude ($\Delta I_{max}/I_0$) as well the duration of the
 475 event (Δt) are illustrated. C) Sketch of Aβ42 fibrils trapping events into a small conical
 476 nanopore (pore 3). Bottom, zoom of a trapping event with different colours representing
 477 the several steps of the trapping.

478 For both fibril samples, the current blockades exhibit a characteristic profile that can be
 479 divided in 4 steps, characteristic of the conical shape nanopore (Figure 6B). In the first
 480 step, the current slowly decreases during several seconds. This response could be assigned

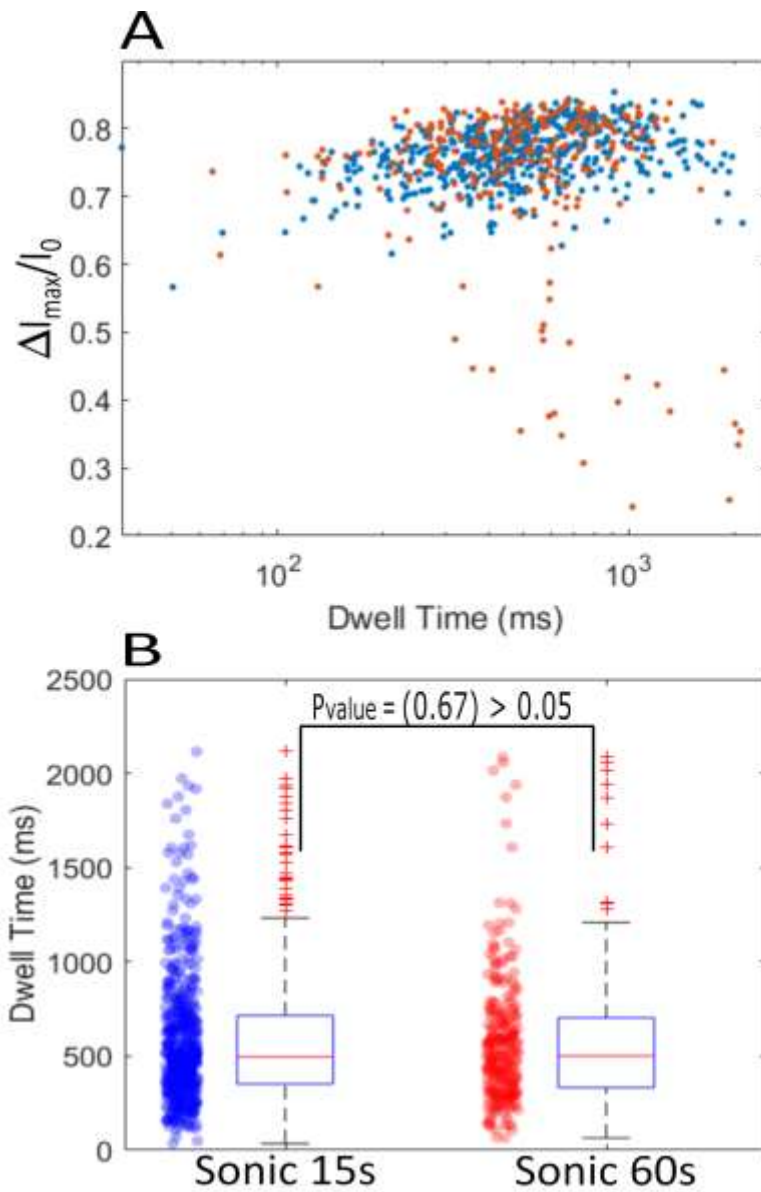
481 to the reorientation of the fibril inside the nanopore to adopt a favourable conformation to
482 translocate. Then, a larger current decrease is observed corresponding to the entrance of
483 the fibril inside the detection zone (yellow in Figure 6B). In the third step, the fibril stays
484 in the detection zone and the current adopts a plateau. Finally, the current quickly increases
485 due to the exit of the fibril outside the nanopore. We notice that the current blockade last
486 several seconds and thus more than one fibrils can be located inside the nanopore at the
487 same time. This is illustrated in Figure SI-13 where the current based line is not recovered
488 after the fibre exit. To avoid any confusion, we did not take into account such event in the
489 analysis. Thus, we have considered in the analysis only the dwell time corresponding to
490 the fibril location inside the detection zone (in blue Figure 6B). In Figure 7A, the $\Delta I_{max}/I_0$
491 vs the dwell time of each recorded event is plotted. First, we observe two populations of
492 current blockade for the sample sonicated 60 s. The larger one centred about 0.76 represent
493 ~91% of event could be assigned to the fibril and the smaller one spread from 0.25 to 0.6
494 (~9 % of events) could be assigned to smaller aggregates (see figure 5C). Conversely, only
495 one population of current blockade was found for the sample sonicated 15 s. The statistical
496 tests show that the two samples sonicated 15 s and 60 s are significantly different (P-value
497 <0.05) (Fig SI-14). However, we notice that the amplitudes of the relative current
498 blockades assigned to the fibrils are similar regardless the sonication time (Figures 6A &
499 SI-13). In order to explain this result, we have to consider the nanopore geometry. Before
500 PEG coating, the diameters of the tip nanopore and the fibrils are close. In this case, the
501 length of the fibrils is not discriminant. For the dwell time, the current blockades have time
502 scales in hundreds milliseconds to 1 second for both samples (Figures 6 A &B). This is
503 two orders of magnitude longer than the dwell time obtained for the non-crowded nanopore

504 (pore 1 coated with PEG 5 kDa, figure 2). This can be explained by the crowded detection
505 zone by the long PEG chain of 20 kDa . As previously demonstrated in experiments and in
506 MD simulations, such environment increases the dwell time. In addition, we cannot
507 exclude an interaction between fibrils and PEG, also explaining why a high voltage (2 V)
508 is required to observe translocation events. Similarly, to the $\Delta I_{\max}/I_0$, the dwell time
509 distributions does not depend on the length of the fibrils. Indeed, there is not significant
510 dwell time distribution for the sample sonicated for 15 s than for the one sonicated for 60
511 s as confirmed by the p-value $\gg 0.05$ (See Figure 7B).

512 We also observe trapping events where a fibril blocks the nanopore for a long time
513 preventing the translocation of other fibrils (Figure 6C). In that case, a fibril is blocked
514 inside the nanopore and cannot fully translocate nor go back due to the high electrophoretic
515 force. The trapping events show numerous fluctuations that can be assigned to different
516 orientation of the fibrils inside the nanopore. However, by applying a voltage of -3 V, the
517 nanopore can be reopened due to the removal of the trapped fibril. To characterize trapping
518 events, we compare the power spectrum density (PSD) between the free- and fibril-
519 blocked-nanopore. An increase of PSD at low frequency is observed when the fibril is
520 trapped (Figure SI-15).

521

522



523

524 Figure 7 : A) Scatter plot representing the $\Delta I_{\max}/I_0$ versus the dwell time for the sample
 525 sonicated 15 s (blue, n = 561) and 60 s (red, n = 268). B) Box plot representing the
 526 differences of dwell time for the samples sonicated 15 s and 60 s (p-value = 0.67).

527 To better understand the interactions between the protein and the nanopore during the
 528 translocation process, we computed pair interaction energies between the amyloid and the
 529 solvent (ion and water) in all the simulations. In Table SI-3 are summarized the averages

530 values of the energies during the simulation time considering the protein centre of mass is
531 located inside or outside the nanopore. We notice only minor modifications of ion pair
532 energy between solvent and the amyloid inside or outside the nanopore. Water interactions
533 appear to be slightly lowered when the amyloid is located inside the nanopore, while still
534 being comprised in our error bars. Interactions of amyloid with PEG and surface cannot be
535 averaged here since the values fluctuate during the translocation process whether the
536 protein adsorbed to the surface. As observed in Figure SI-16, PEG interactions with the
537 amyloid depends on both the amyloid size and the PEG density. In the low PEG density ,
538 we observe for 2NAO a progressive decrease of the pair energies while the amyloid enters
539 in the pore. Then it reaches a plateau about $-120 \text{ kcal mol}^{-1}$ before returning to 0 as soon as
540 the protein leaves the nanopore. We observe the same trend with 5OQV. However, part of
541 a PEG molecule is still adsorbed on the amyloid even after it left the pore. For the high
542 density PEG, we notice stronger interactions with the amyloid, up to $-500 \text{ kcal mol}^{-1}$ for
543 2NAO and $-800 \text{ kcal mol}^{-1}$ for 5OQV. This is explained by the increase of the number of
544 PEG around the amyloid increases. This is consistent with the slowing down the
545 progression of the amyloid through the pore as shown in Table 1. We cannot establish with
546 precision the lowest pair energy level with the PEG since the curve has not reached their
547 equilibrium. Surface interaction is found to be lower for the high density of PEG, as the
548 latter hide the nanopore surface (Figure SI-17). For the low density of PEG, we observe
549 strong interaction for 5OQV reaching $-500 \text{ kcal mol}^{-1}$ due to the adsorption of amyloid
550 onto the nanopore surface from 18 to 44 nanoseconds in our simulation. To sum up, our
551 simulation shows that the protein interacts more with the solvent than with the other PEG,
552 and surface. In addition, the protein/solvent interactions do not depend on the PEG density.

553 However, the amyloid/PEG and amyloid/surface interactions cannot be neglected since
554 represent 1 to 5 % of the interaction with the solvent. This suggests that the amyloid/PEG
555 interaction appears to be the main modified values during the diffusion of the fibrils.

556

557 4. Conclusion

558 In summary, we report detection of A β 42 fibrillar samples with different length
559 distributions by tuning the shape of track-etched nanopores and PEG functionalization. Our
560 finding that adding EGCG during A β 42 amyloid formation leads to a different fibril
561 morphotype with an altered fibril length and fragmentation stability is conceptually
562 attractive, and was used here for our nanopore detection strategy to characterize various
563 well-defined fibril populations.

564 First we found that conical nanopores coated with PEG 5 kDa allow a good
565 discrimination of the fibril size by using $\Delta I_{max}/I_0$. We then demonstrate that the bullet shape
566 nanopore enhances the amplitude of the current by a factor of 3. In addition, the dwell time
567 increases by one order of magnitude. Therefore, we show that by exploiting both these
568 parameters, the $\Delta I_{max}/I_0$ and the dwell time, fibril size can be well discerned. The use of
569 conical nanopores with a tip aperture crowded with large PEG revealed enhanced relative
570 current blockades upon fibril translocation, with no significant differences on both the
571 $\Delta I_{max}/I_0$ and the dwell time between two different-sized fibril samples. Using MD
572 simulations, we then confirmed strong interactions of the fibrils with the PEG
573 functionalization of the conical nanopore, reflected by a larger dwell time.

574 We demonstrate here in this work that the track-etched nanopore allows to detect and
575 discriminate of fibrils. Indeed, we can control crowding and tune the geometry of the

576 nanopore by PEG functionalization. Thus, it becomes possible to enhance the amplitude of
577 relative current blockade and/or the dwell time. Considering that, such approach opens the
578 way for further investigation of the different species involved in the amyloidogenesis
579 pathway.

580

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589

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