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1 Conical nanopores highlight the pro-aggregating
2 effects of pyrimethanil fungicide on A β (1-42)
3 peptides and dimeric splitting phenomena

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16 **Abstract**

17 The A β (1-42) aggregation is a key event in the physiopathology of Alzheimer's disease (AD).
18 Exogenous factors such as environmental pollutants, and more particularly pesticides, can
19 corrupt A β (1-42) assembly and could influence the occurrence and pathophysiology of AD.
20 However, pesticide involvement in the early stages of A β (1-42) aggregation is still unknown.
21 Here, we employed conical track-etched nanopore in order to analyse the A β (1-42) fibril
22 formation in the presence of pyrimethanil, a widely used fungicide belonging to the
23 anilinopyrimidine class. Our results evidenced a pro-aggregating effect of pyrimethanil on
24 A β (1-42). A β (1-42) assemblies were successfully detected using conical nanopore coated with
25 PEG. Using an analytical model, the large current blockades observed (>0.7) were assigned to
26 species with size close to the sensing pore. The long dwell times (hundreds ms scale) were
27 interpreted by the possible interactions amyloid/PEG using molecular dynamic simulation.
28 Such interaction could leave until splitting phenomena of the dimer structure. Our work also
29 evidences that the pyrimethanil induce an aggregation of A β (1-42) mechanism in two steps
30 including the reorganization prior the elongation phase.

31 **Keywords:** amyloid, nanopore, resistive pulse, fungicide, A β (1-42), track-etched, lag phase,

32 **1. Introduction**

33 The assembly of normally soluble proteins into an amyloid fold has become the dominant
34 model that explains the pathogenesis of numerous neurodegenerative disorders, including
35 Alzheimer's (AD) and Parkinson's diseases (Chiti and Dobson, 2017; Ke et al., 2020). This
36 critical misfolding pathway, called amyloidogenesis, is guiding the development of potential
37 treatments since it generates structurally different protein assemblies that are thought to be
38 responsible for the cellular toxicity (Soto and Pritzkow, 2018). Although unexpectedly diverse
39 and complex, the amyloid fold is structurally characterized by a typical cross- β fibril
40 architecture and is often considered to be energetically the most favourable protein state (Gazit,

41 2002). Nevertheless, amyloids are dynamic, with monomers/oligomers association and
42 dissociation from their ends and surface (Carulla et al., 2010; Jahn and Radford, 2008). In vitro,
43 kinetic growth curves for A β (1-42) fibril formation starting from pure monomer display a
44 sigmoidal profile, where after a first slow amyloid size increase (lag phase), the aggregates start
45 to growth (growth phase) until saturation is reached. Oligomeric species populated during the
46 lag phase are heterogeneous and rapidly evolve to amyloid fibrils during the growth phase, in
47 which fibril mass increases with time to an equilibrium plateau (Michaels et al., 2018). There
48 is compelling evidence that different physicochemical factors can influence the duration of the
49 lag phase. The search for a trigger event leading to an accelerated A β (1-42) fibril formation has
50 mostly been investigated from the perspectives of the properties of A β itself (i.e., mutations,
51 truncations), but marginally from abnormal interactions with exogenous synthetic molecules
52 (McLaurin et al., 2000).

53 Indeed, environmental pollutants are now suspected to play a role in the aetiology or
54 incidence of neurological disorders (Chin-Chan et al., 2015). Epidemiological studies revealed
55 that occupational exposure of farmers to organophosphates has been associated with an
56 increased risk to develop Alzheimer's disease (Hayden et al., 2010). Three fungicides belonging
57 to the anilinopyrimidine class, i.e. cyprodinil, mepanipyrim and pyrimethanil are widely used
58 for crop preservation, as well as for long-term storage of fruits and vegetables. Many reports
59 have in the past established their presence in our environment, food as well as in baby food
60 jars(Nougadère et al., 2020; Sánchez-Santed et al., 2016) . Recently, enhanced A β aggregation,
61 microgliosis and neuronal loss have been observed after treatment of AD transgenic mouse
62 model with residual amounts (0.1 μ g/L in drinking water for 9 months) of a cocktail of 3
63 fungicides, cyprodinil, mepanipyrim, and pyrimethanil (Lafon et al., 2020). A β pro-aggregating
64 activity appears to be mediated by the direct interaction of fungicides with A β (1-42).
65 Nevertheless, the influence of such chemical agents in A β (1-42) assembly, and

66 amyloidogenesis, as well as their interplay with specific subsets of A β (1-42) assemblies remain
67 to be determined.

68 While the underlying molecular events occurring during A β (1-42) fibril growth are now
69 widely recognized, less is known about the transient oligomers formed at earlier stages
70 especially during the lag phase. The reason is that conventional techniques do not provide
71 sufficient resolution to detect and characterize these intermediate species in bulk assays.
72 Therefore, an experimental technique is needed allowing for high-sensitive real-time detection
73 and characterization of early stages of A β (1-42) assembly. Recently, the use of nanopore
74 technology demonstrated very promising results thanks to the possibility to obtain information
75 about the protein aggregate population under continuous measurement(Houghtaling et al.,
76 2018b). Biological nanopores were widely used for protein sensing. They were shown to be
77 able to detect minor differences in peptide sequences (Asandei et al., 2017; Huang et al., 2019;
78 Ouldali et al., 2020; Piguet et al.) and to analyse the protein folding/unfolding process (Di
79 Marino et al., 2015; Merstorf et al., 2012; Oukhaled et al., 2007; Rodriguez-Larrea and Bayley,
80 2013). They were used to investigate the prion protein and α -synuclein misfolding (Madampage
81 et al., 2012; Stefureac et al., 2008; Tavassoly and Lee, 2012), A β peptide aggregation (Bonome
82 et al., 2019; Wang et al., 2011) or binding with metal ion (Asandei et al., 2013; Asandei et al.,
83 2014). The A β (1-42) aggregation was evidenced by α -hemolysin as bumping event, making it
84 impossible to obtain information about the amyloid size (Wang et al., 2011). The solid-state
85 nanopores have advantages of robustness, reusability and scalable diameter (Lepoitevin et al.,
86 2017). They allow the characterization of conformational fluctuations of proteins (Waduge et
87 al., 2017), their interactions with nanoparticles (Coglitore et al., 2018; Coglitore et al., 2019) as
88 well as protein aggregation. The unmodified SiN nanopore allows detection of lysozyme
89 oligomers (Balme et al., 2016). The glass nanopipettes are also used for amyloid detection as
90 shown for lysozyme (Martyushenko et al., 2015), α -synuclein (Chau et al., 2020) and A β (1-42)

91 (Yu et al., 2019). However, the nanopore functionalization is required to prevent the nanopore
92 fouling in the case of A β (1-40) (Yusko et al., 2011) or α -synuclein (Hu et al.), and to allow
93 discrimination of oligomers (Yusko et al., 2012). The functionalization with PEG is efficient to
94 discriminate different types of protein aggregates (Giamblanco et al., 2018b) while at the same
95 time increasing the nanopore lifetime (Roman et al., 2017). The limitation of solid-state
96 nanopores and glass nanopipettes is that they do not allow to differentiate aggregates and
97 protofibrils larger than the nanopore diameter. Indeed, when the aggregates are larger than the
98 pore, only bumping events are observed making the size discrimination impossible. To
99 overcome this problem, our group proposed the use of conical track-etched nanopore
100 (Giamblanco et al., 2018a). The latter are more used for their ionic-diode properties than single
101 molecule sensing (Ma et al., 2020; Zhao et al., 2017). However, by combining this geometry
102 and a very long lifetime, they are more convenient to monitor protein aggregation kinetic
103 (Giamblanco et al., 2018a; Giamblanco et al., 2020a) as well as their enzymatic degradation
104 (Giamblanco et al., 2020b).

105 To investigate the influence of extrinsic factors on A β (1-42) assembly we selected the
106 fungicide pyrimethanil, which belongs to the anilinopyrimidine class. In this work, the A β (1-
107 42) amyloidogenesis in the absence and presence of pyrimethanil was monitored by a thioflavin
108 T (ThT) binding assay, and characterized using conical track-etched nanopore technology as an
109 *ex-situ* assay. Using this up-to-date approach, we focused mainly on the lag phase that was
110 determined during the amyloid fibril formation. To do so, we produced several nanopores with
111 a tip diameter adapted to the degree of aggregate maturation, and analysed the A β (1-42) species
112 present from a reaction mixture at different times using resistive pulse experiments. The
113 experimental data of current blockades were compared with a theoretical model and with the
114 results of molecular dynamics simulations.

115 **2. Materials and methods**

116 **2.1. A β (1-42) aggregation and characterization**

117 ***Preparation of monomeric A β (1-42)***. A β (1-42) peptides (ERI Amyloid Laboratory LLC,
118 Oxford, CT,USA)) were maintained in a monomeric state using the protocol described in Serra-
119 Batiste et al. (Serra-Batiste et al., 2016). Briefly, A β (1-42) peptides were dissolved in a 6.8 M
120 guanidine thiocyanate solution (Sigma-Aldrich) at a concentration of 8.5 mg mL⁻¹. The solution
121 was then sonicated for 5 min at 52 °C, and diluted with ultrapure water to reach a final
122 concentration of 5 mg mL⁻¹ of A β (1-42) peptides and 4 M of guanidine thiocyanate. Solution
123 was centrifuged at 10,000 g for 6 min at 4°C. The collected supernatant was filtered (PVDF,
124 0.45 μ m) and then injected into a Superdex 75 Increase 10/300GL column (GE Healthcare Life
125 Science) previously equilibrated with 10 mM sodium phosphate buffer pH 7.4. Purification was
126 performed with a 0.5 mL min⁻¹ flow to collect the peak attributed to monomeric A β (1-42). The
127 A β (1-42) peptides concentration was determined with a NanoDrop 8000 spectrophotometer
128 (Thermo Scientific). The aliquots of peptides were stored at -20 °C.

129 ***Preparation and characterization of A β (1-42) aggregates***. A β (1-42) stock solution was
130 diluted to 30 μ M in a 10 mM sodium phosphate buffer, pH 7.4 and left to aggregate in low-
131 binding Eppendorf tubes for a final volume of 600 μ L. Experiments with pyrimethanil were
132 conducted under the same conditions, except that fungicide was added from a filtered (PVDF,
133 0.22 μ m) stock solution to get a 1:15 molar ratio (A β (1-42): pesticide), with a final
134 concentration of DMSO of 1% (v/v). To keep the identical conditions, an equal volume of
135 DMSO was added to the control tube. Tubes were arranged vertically and incubated at 25 °C
136 under quiescent state. The fibril formation was monitored by a thioflavin T (ThT) binding assay.
137 Briefly, 20 μ L aliquots were withdrawn at specific times and mixed with 14 μ L of 142 mM
138 GlyNaOH buffer, pH 8.3 and 6 μ L of 100 μ M of ThT in a 96-well plate of black polystyrene
139 with a clear bottom coated with a PEG (Thermofisher Scientific). ThT fluorescence of each
140 sample was measured (λ_{ex} = 445 nm and λ_{em} = 485 nm) in a Fluoroskan Ascent microplate

141 fluorimeter (Thermofisher Scientific). Aggregation profiles were generated with GraphPad
142 Prism software. Curves were fitted using the built-in sigmoidal fit. Each kinetic trace was fitted
143 independently to extract the time required to reach 50% of the maximum fluorescence intensity,
144 known as t_{50} .

145 **2.2. Nanopore production and characterization**

146 Single tracks were produced by Xe irradiation (8.98 MeV u^{-1}) of PET film ($6 \mu\text{m}$,
147 Goodfellow) at GANIL (SME line, Caen, France). The activation of the track was performed
148 by a UV exposition of 8 h for the tip side and 11 h for the base side (Fisher bioblock;
149 VL215.MC, $\lambda=312 \text{ nm}$) before the chemical etching process. The etching of the conical
150 nanopore was performed under asymmetric conditions as previously reported (Lepoitevin et al.,
151 2016). After the nanopore etching, the film was stabilized overnight in $18 \text{ M}\Omega$ water. The
152 approximate tip diameter was obtained by conductance measurement. After the chemical
153 etching, the current-voltage (I-V) curve was measured at pH 7. Then the nanopore was
154 functionalized with $\text{PEG}_{5k}\text{-NH}_2$ (Nanocs, PG1-AM-5K) using 1-ethyl-3-(3-
155 dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma Aldrich, E7750) as
156 previously reported in order to prevent the unspecific adsorption and improve the
157 nanopore lifetime³⁵. The success of the grafting was confirmed by the decrease of I-V curve
158 slope due to the replacement of COO^- by the PEG which diminished the diameter of the
159 nanopore (see figure S1). The estimation of nanopore size was performed using quasi-1D model
160 (see supporting information section 1)

161 **2.3. Detection of $\text{A}\beta(1-42)$ aggregates using resistive pulse**

162 The nanopore functionalized with PEG moieties was mounted between two compartments of
163 a Teflon cells filled with PBS 1X aqueous solution (pH = 7.2). The $\text{A}\beta(1-42)$ at different
164 aggregation times (from 0 to 72 h) in presence or not of pyrimethanil was added to the base side
165 to reach a concentration of 30 nM (monomer equivalent). The resistive pulse experiments were

166 performed using a patch-clamp amplifier (EPC 10, HEKA electronics, Germany) with Ag/AgCl
167 electrodes. A voltage of 1 V was applied to the working electrode located in the tip side
168 compartment to drive the negatively charged A β (1-42), (isoelectric point of 5.5) to pass through
169 the nanopore from the base to the tip side. The ionic currents were recorded at 50 kHz. The
170 signal was filtered at 5 kHz by a Bessel filter. The resistive pulse detection was recorded on the
171 fly. The current traces were further analysed to detect events using lab-made software “Peak
172 Nano Tools” developed using Labview. First the signal was filtered using Butterworth filter
173 1 kHz order 1. The threshold for the event detection was defined as follows: (i) correct the
174 baseline using a Stavinsky-Golay filter (ii), define the noise levels by the global standard
175 deviation methods (iii) define the threshold. In this work, the threshold has been fixed at about
176 4σ (where σ is the standard deviation of the signal after baseline correction).

177 **2.4. Molecular dynamic simulation**

178 *Description of the protocol* Classical all-atom MD simulations were performed using the
179 NAMD.2.12 package(Phillips et al., 2005) . The different systems were solvated in a water box
180 large enough to prevent the interaction between the central part (the conical pore) and its
181 neighbouring periodic cells. KCl ions (at a concentration of 1 M) were added to the water
182 (simulated using the TIP3P model) to reproduce the experimental environment. CHARMM36
183 (Brooks et al., 2009; Lee et al., 2015) force-field optimization parameters were used in all
184 simulations. During the simulations, the system temperature and pressure were kept constant at
185 300 K (Langevin dynamics) and 1 atm (Langevin piston), respectively. The long-range
186 electrostatic forces were evaluated using the classical particle mesh Ewald (PME) method with
187 a grid spacing of 1.2 Å, and a fourth-order spline interpolation. The integration time step was
188 equal to 1 fs. Each simulation employed periodic boundary conditions in the three directions of
189 space.

190 *Description of the conical nanopore.* To build the conical solid-state nanopore ($R_{tip} = 3$ nm,
191 $R_{base} = 3.5$ nm, length=14.8 nm) several carbon nanotube sections of different radii were
192 associated and centred along the nanopore axis. To model the chemical structure of PET
193 nanopore used in the experiments, one third of the carbon atoms constituting the nanopore were
194 randomly configured as oxygen atoms. Partial charges, positive for carbon and negative for
195 oxygen atoms, were added while global neutrality of the nanopore was conserved. Each section
196 was then modified according to the experimental functionalization of the conical nanopore by
197 grafting 30 hydroxyls functions (0.10 M) to randomly picked carbon atoms. Then, three
198 PEG 5K were added (1.15 M), as in the experiments. To place the PEG, a position of linkage
199 was chosen with a random generator number. Nevertheless, to orient each molecule, we took
200 into account the position of each one in order to avoid the superposition of the different
201 structures. Finally, the functionalized nanopore was placed between 2 reservoirs of dimensions
202 equal to $133 \times 133 \times 68 \text{ \AA}^3$ and solvated with K^+ and Cl^- ions concentration equal to 1 M. The
203 reservoir size was chosen to be around twice the size of the conical-tube part of the nanopore.
204 The complete system dimensions are $133 \times 133 \times 285 \text{ \AA}^3$, for a total of around 320.000 atoms,
205 detailed as such: 24.000 for the nanopore, 2.400 for PEGs, 3.700 for ions and 290.000 for water.
206 Once the complete functionalized system was obtained, it was optimized following three
207 successive procedures. First, we minimized the energy of the total system at 0 K. Then, the
208 system was progressively heated until reaching a temperature equal to 300 K. Finally, the
209 system was left to evolve at the NPT ensemble, and physical observables were calculated using
210 time averages. During all simulations, every atom constituting the conical nanopore was kept
211 fixed. This system was relaxed for 30 ns, allowing for PEG to relax around the surface of the
212 nanopore. For further computations dealing with ionic current calculations, various electric
213 fields were applied. Those simulations were performed in the NVT ensemble. Once run, the
214 determination of the ionic current was performed according to the procedure described in Ref

215 (Aksimentiev and Schulten, 2005) by following each ion in the nanopore during its
216 displacement.

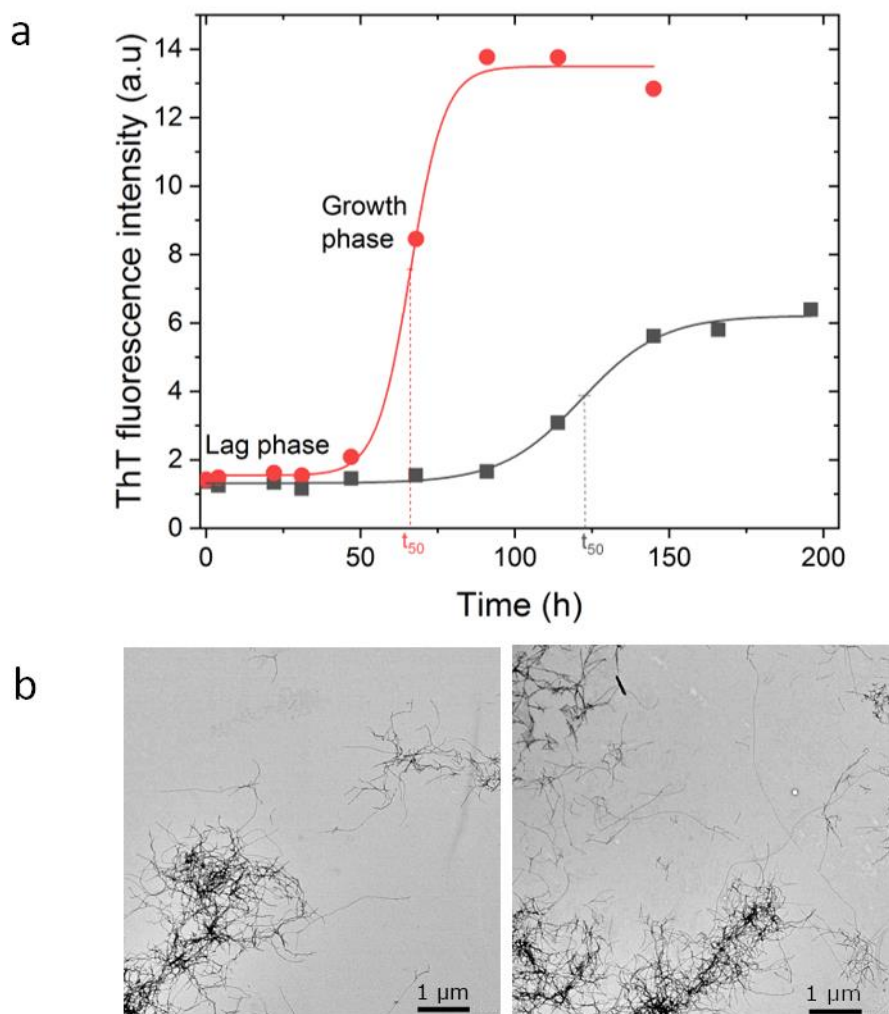
217 *Description of the simulated different amyloids. 5OQV/2NAO/2BEG:* Three types of A β (1-
218 42) amyloid arrangements were generated and studied by simulations. To the previously
219 described 5OQV(Gremer et al., 2017) and 2NAO(Wälti et al., 2016)dual subunit amyloid
220 structure, we also studied a single subunit (PDB id = 2BEG(Lührs et al., 2005)) in order to see
221 its role on the current perturbation inside the nanopore. First, we were interested in the
222 behaviour of small amyloid structures (i.e., 2BEG) in the conical nanopore. Structures used in
223 this case were extracted from RCSB Protein Databank, relaxed in water and 1M KCl ions for
224 10 ns and then inserted inside the relaxed functionalized nanopore. Once it was shown that
225 simulating translocation under a voltage was possible, we simulated the translocation of the
226 bigger amyloid structures (2NAO and 5OQV). For each simulation run, the relaxed 2NAO or
227 5OQV was placed at the entrance of the previously described functionalized nanopore, free of
228 water and ions. Water and ions were then added into the systems and relaxed. Note that for the
229 relaxed 2BEG amyloid, we started the simulation with the amyloid inside the nanopore.

230 **3. Result and discussion**

231 **3.1. Impact of pyrimethanil on the aggregation**

232 The aggregation kinetics starting from pure monomeric A β (1-42) was characterized by using
233 the reporter dye thioflavin T, which undergoes an enhancement of quantum yield when bound
234 to β -sheet structure of fibrils (Figure 1A). The addition of pyrimethanil efficiently accelerated
235 the reaction by decreasing to 50% the half-time of aggregation (t_{50}) (from about 122 to 66
236 hours). The fluorescence intensity of ThT at the post-transition plateau in the presence of
237 pyrimethanil was significantly higher (about a two-fold increase), compared to that obtained
238 using A β (1-42) fibrils alone. This supports the idea that pyrimethanil facilitates a
239 conformational rearrangement leading to different fibril morphotypes. However, no major

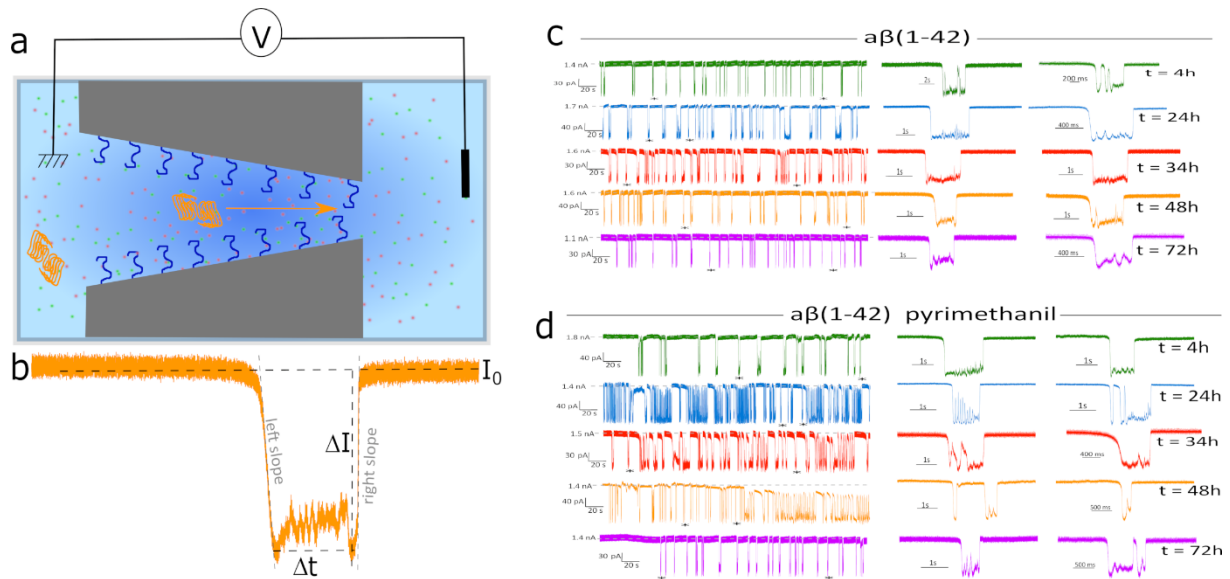
240 macrostructural changes induced by pyrimethanil treatment were revealed by transmission
241 electron microscopy (Figure 1b-c).



242
243 **Figure 1: Effect of pyrimethanil on the kinetics of A β fibrils formation. (a) A β (1-42) monomers (30 μ M)**
244 **were incubated with pyrimethanil (750 μ M) (red curve), or with an equivalent volume of DMSO (1%) (black**
245 **curve) with a ratio 1:15. ThT fluorescence intensity was measured once a day (a.u., arbitrary units). The**
246 **different values obtained were plotted. The sigmoidal fits were done using Boltzmann equation ($I = I_0 +$**
247 **($I_0 - I_F$)/1 + exp($t_{50} - t/a$)) where I, I_0 and I_F are the fluorescence intensity at time t, at the initial and**
248 **final plateaus respectively, and t_{50} the half-time of aggregation. (b) Negative-stained transmission electron**
249 **micrographs of A β (1-42) fibrils formed in the absence (left panel) or presence (right panel) of pyrimethanil.**

250 3.2. Investigation of the lag phase using nanopore

251 To further characterize the pro-aggregating effect of pyrimethanil on A β (1-42) during the lag
252 phase of amyloidogenesis the aliquots were withdrawn at different time intervals and analysed
253 using conical track-etched nanopores. To do so, we used a conical nanopore functionalized with
254 PEG as previously reported(Giamblanco et al., 2018a). The characterization of nanopore using
255 quasi-1D model for pore resistance gives a pore tip radius $r_t \sim 4.7$ nm while the pore base
256 radius $r_b = 170$ nm was determined by SEM (See Supplementary Information S1). The detection
257 of A β (1-42) aggregates was performed using resistive pulse methods from the base to the tip
258 side (Figure 2a). From the current traces, we detected events characterized by the relative
259 current blockade noted $\Delta I/I_0$, the dwell time Δt , both the left and right slope and the variance of
260 the close current (Figure 2b). In Figure 2, the current trace recorded for A β (1-42) at various
261 incubation times (from 4 h to 72 h) with pyrimethanil or not are reported. First, we observed
262 the current blockade for all the samples. This means that after 4h in presence or absence of
263 pyrimethanil, A β (1-42) aggregates are detected. The zoom of certain current blockades reveals
264 large fluctuations of the closed current levels that can be assigned to the reorientation of an
265 aggregate inside the detection zone of the nanopore. During the experiment, we also observe
266 events where the base line level is not reached while the event is supposed to be finished (see
267 figure 2 d, orange line). This could have two interpretations (i) an aggregate is adsorbed close
268 to the narrow aperture or (ii) a large aggregate is trapped inside the nanopore. Because the origin
269 of such events is not clear, we did not consider these events in the further analysis. For each
270 event, we analysed both the left and the right slope (see SI-2, figure S3). For all recorded events,
271 the values of the left slope corresponding to the beginning of the event are one order of
272 magnitude larger than the right one that corresponds to the aggregate exit from the nanopore.
273 Such dissymmetry is a typical feature of the track-etched nanopore presenting a conical shape.



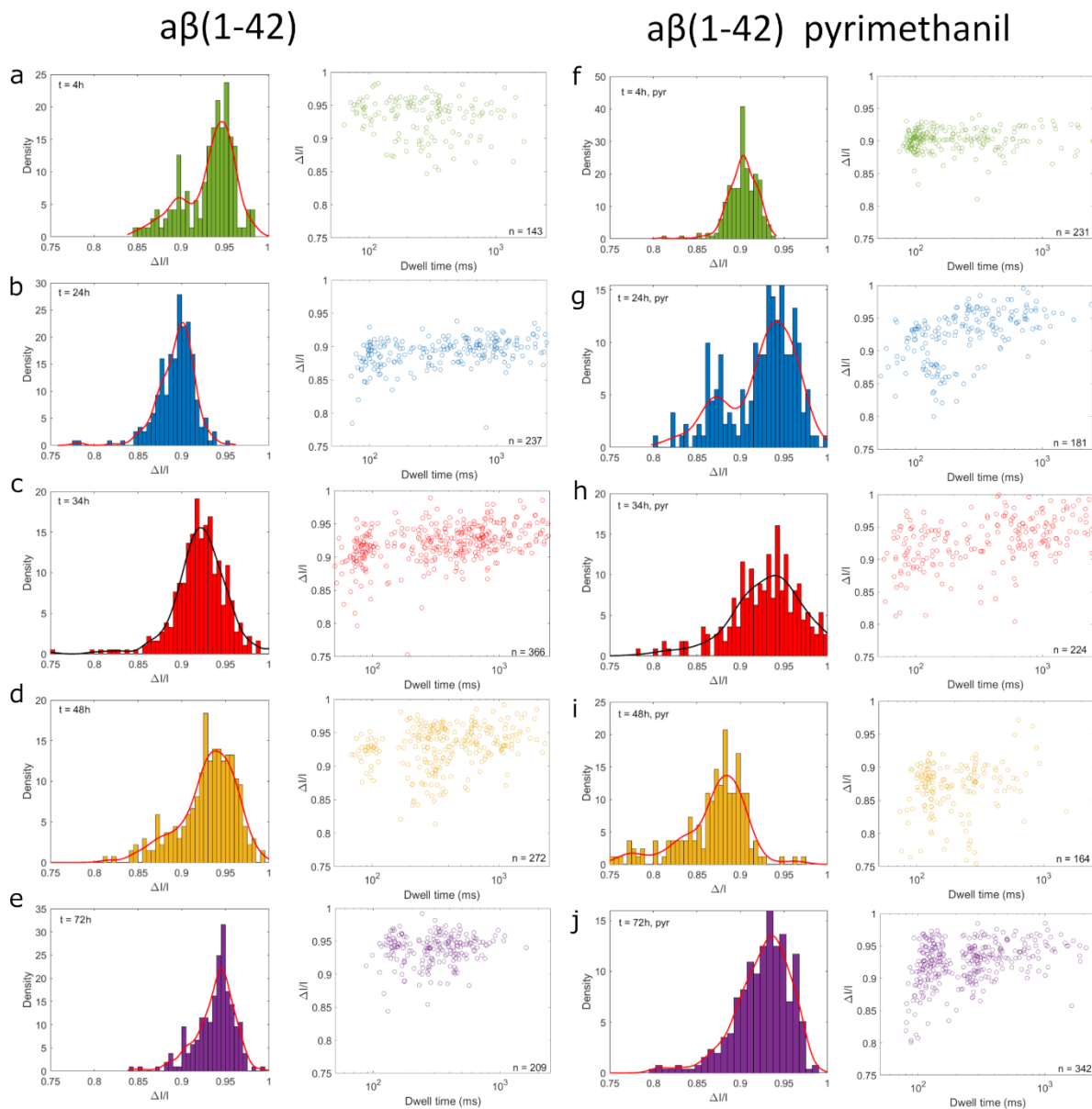
274

275 **Figure 2: (a) Sketch of nanopore experiments. Aβ(1-42) after incubation 72 h with and without pyrimethanil**
 276 **(b) illustration of the parameters measured for each current blockade. Examples of current traces recorded**
 277 **during resistive pulse experiments for Aβ(1-42) after incubation without (c) and with pyrimethanil (d) for**
 278 **various times. The experiments were performed using nanopore $r_t \approx 4.7$ nm. On the right side, example of**
 279 **current blockade is reported.**

280 From the current traces, we detected events characterized by the relative current blockade
 281 noted $\Delta I/I_0$ and the dwell time Δt . In Figure 3, both the distribution of $\Delta I/I_0$ and the event map
 282 recorded for Aβ(1-42) after 4 h, 24 h, 34 h, 48 h, 72 h, incubation without or with pyrimethanil
 283 are reported. Without pyrimethanil and after 4h, the distribution is bimodal centred to 0.90 and
 284 0.94 ± 0.04 evidencing the presence of two distinct populations in the sample. After 24h, the
 285 distribution becomes monomodal centred to 0.90 ± 0.04 . Interestingly, the larger $\Delta I/I_0$
 286 distribution disappears. This result is counter-intuitive. Indeed, we expected that with time the
 287 aggregate should be larger. However, we observe an increase of the dwell time distribution
 288 (Figure SI-5). This means that the assemblies occupy less space inside the nanopore but
 289 translocate more slowly. This suggests at this stage a reorganization of the aggregate. After 34h
 290 and 48h, we observe that the amplitude of the relative current blockade increases and their
 291 distribution is wider (0.92 ± 0.06 and 0.93 ± 0.06 respectively). In addition, the distribution of the

292 dwell time is more spread and reveal two clusters. The first population exhibits a dwell time >
293 105 ms, while the second one is spread from 110 ms to 2500 ms. This suggests that the
294 population of oligomers is more and more heterogeneous. After 72 h, the $\Delta I/I_0$ distribution is
295 shaper, reaching 0.95 ± 0.04 . On the other hand, the dwell time distribution is also more compact
296 since only one population is obtained. Concerning the distribution of $\Delta I/I_0$ and the events map
297 for the for A β (1-42) incubated in presence of pyrimethanyl we can report that after 4 h of
298 incubation, the distribution of $\Delta I/I_0$ is centred to 0.90 ± 0.04 . After 24 h and 34 h, the distributions
299 $\Delta I/I_0$ are wider and centred 0.94 ± 0.06 and 0.94 ± 0.09 . We also observe that the dwell times
300 distributions are more spread, suggesting heterogeneity in the sample. However, compared to
301 the sample without pyrimethanyl, the presence of two distinct populations of dwelling time is
302 not evidenced. After 48 h, the distribution centre of $\Delta I/I_0$ decreases to 0.88 ± 0.06 . On the other
303 hand, the dwell time distribution is centred to lower values. This clearly evidences the presence
304 of smaller assemblies. It is interesting to notice that 48 h corresponds to the beginning of the
305 growth phase (figure 1a) determined by the ThT test. After 72 h, the aggregation process is
306 clearly under the growth phase, the distribution shifts toward larger value of $\Delta I/I_0$ (0.93 ± 0.07)
307 and longer dwell time. Our nanopore experiments reveal that the evolution of the population of
308 A β (1-42) follows two different trends. Without the pyrimethanyl, after the reorganization
309 occurring between 4 h and 24 h, the A β (1-42) aggregates are more and more polydispersed with
310 the incubation time. This is also the case for the sample incubated with pyrimethanyl before the
311 starting of the growth phase where the aggregate structures become more ordered adopting a β -
312 sheet organization. After 48 h of incubation with pyrimethanyl, the presence of smaller
313 assemblies revealed by nanopore experiments is consistent with a quaternary structural
314 convergence, by reducing the parental polydispersity, and generation of smaller assemblies.
315 These results led us to conclude that conformational rearrangements and protomer exchanges,

316 i.e., dissociation/association events, take place within the initial heterogeneous population of
 317 assemblies(Dear et al., 2020b; Dear et al., 2020a; Michaels et al., 2020).



318
 319 **Figure 3: histograms of relative current blockade without (a-e) and with pyrimethanil (f-j) and**
 320 **corresponding event maps of current blockade parameters induced by Aβ(1-42) after various incubation**
 321 **time (4 h green, 24 h blue, 34 h red, 48 h orange, 72 h violet), the lines are the fit using nonparametric law**
 322 **to evaluate the centre of the distribution. The experiments were performed using nanopore $r_t = 4.7$ nm.**

323 We then performed a theoretical analysis of the expected blockade to estimate the size of
 324 Aβ(1-42) aggregate. In general, the current blockade induced by a particle dwelling into a
 325 nanopore depends on the particle size, shape, position, orientation, surface charge and

326 electrolyte solution (Houghtaling et al., 2018a). In nanofluidic systems, different
327 theoretical/computational techniques are commonly used to describe the current blockade and
328 the translocation process (Chinappi and Cecconi, 2018; Qiu et al., 2016). Here, we start our
329 analysis using a theoretical model while in the next section, we discuss molecular dynamics
330 simulations.

331 One evidence shown in Fig 2 is that the current blockades are always relatively high, i.e.
332 $\Delta I/I_0 \simeq 0.85 - 0.95$. Two questions naturally arise, i) what is the shape of the amyloid
333 aggregates responsible for such high blockades? and ii) why, despite the expected
334 polydispersity of the sample (in a growing process, we expect that different populations, from
335 monomers to large aggregates are present simultaneously) small blockades (e.g., $\Delta I/I_0 > 0.7$)
336 are not observed?

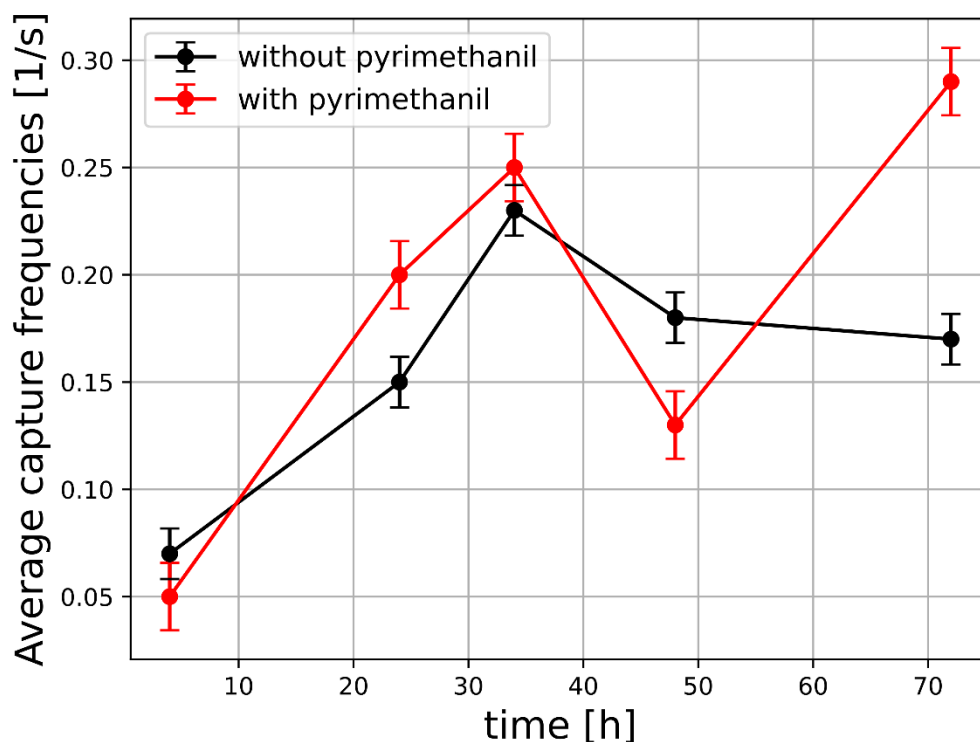
337 To answer to these questions we employed a quasi-1D model that allows estimating current
338 blockades associated with the presence of a particle partially blocking the pore (DeBlois and
339 Bean, 1970; Di Muccio et al., 2019; Giambianco et al., 2018a). Assuming that the aggregates
340 have a spherical shape with radius r_a , our model predicts that the blockade ($\Delta I/I_0 \simeq 0.85 -$
341 0.95) are due to aggregate radii close to the nanopore tip radius, i.e. $r_a \simeq r_t$, see section 3 of SI
342 for details on the physical model. This answers to the first question: the experimentally
343 observed blockades correspond to particles of radius $r_a > 4.5$ nm that occupies the pore tip.
344 The present model is developed for spheres, similar analytic results can be obtained for other
345 simple shapes (e.g. short cylinders or ellipses). The different peaks observed experimentally
346 (see e.g. figure 2a or figure 2g) can be ascribed to particles with slightly different shape and size.

347 Moreover our model predicts that amyloid aggregates with smaller radius would result in
348 much smaller blockades, e.g., an aggregate of radius 4 nm would gives $\Delta I/I_0 \simeq 0.2$. In
349 principle, such blockade should be detected by our nanopore sensing system. Hence, the fact
350 that such small blockades are not observed deserve an additional explanation. In our opinion,

351 the most reasonable explanation is that such small aggregates exist in the solution and they
352 actually translocate through the pore. However their translocation is so fast that the signal is not
353 recorded. To support this interpretation, we estimate the dwell time of an aggregate as a function
354 of its radius, see section S4 of SI. The dwell time predicted using only electrophoresis as driving
355 force is much smaller than the observed one. This implies that aggregates are further slowed
356 down by interaction with the pore wall. This interaction is effective only for aggregate of the
357 same size of the pore tip. In summary, our model suggests the following interpretation:
358 aggregates of different sizes are present in the solution. The ones are smaller than the pore tip,
359 $r_a < r_t$, translocate too fast to be detected. The ones have a size similar to the pore, $r_a \sim r_t$, are
360 slowed down by the interaction with pore tip wall and they provide an almost complete blocking
361 of the current $\Delta I/I_0 > 0.85$. Larger aggregates, $r_a > r_t$, could exist in the solution and the
362 resulting blockage would be even larger. Nevertheless, the analysis of the left and right slopes
363 of the blockade events suggest that all the events are associated to translocations (see Figure 2
364 and Fig S2). Consequently, larger aggregates probably bump at the pore surfaces without
365 reaching the sensing region.

366 In the light of this interpretation, the pore can also be used as a counter for aggregate of size
367 $r_a \approx r_t$. The event frequency is reported in Figure 4. In the lag phase $t < 40$ h, the event
368 frequency increases with time as expected in an aggregation process. This increase is slightly
369 more pronounced in the experiments with pyrimethanil, suggesting a possible positive effect of
370 pyrimethanil in the first phase of aggregation, coherently with the fluorescence data in Figure
371 1. With pyrimethanyl, we observe a decrease of the capture rate at 48 h of incubation. This can
372 be explained by a decrease of the aggregate size and thus a part of these aggregates would
373 become too small to be detected by nanopore. Interestingly, the evolution of the capture rate
374 and the size of aggregate suggest that the pyrimethanyl induce a mechanism of aggregation in
375 two steps due to a reorganization and/or fragmentation phenomena.

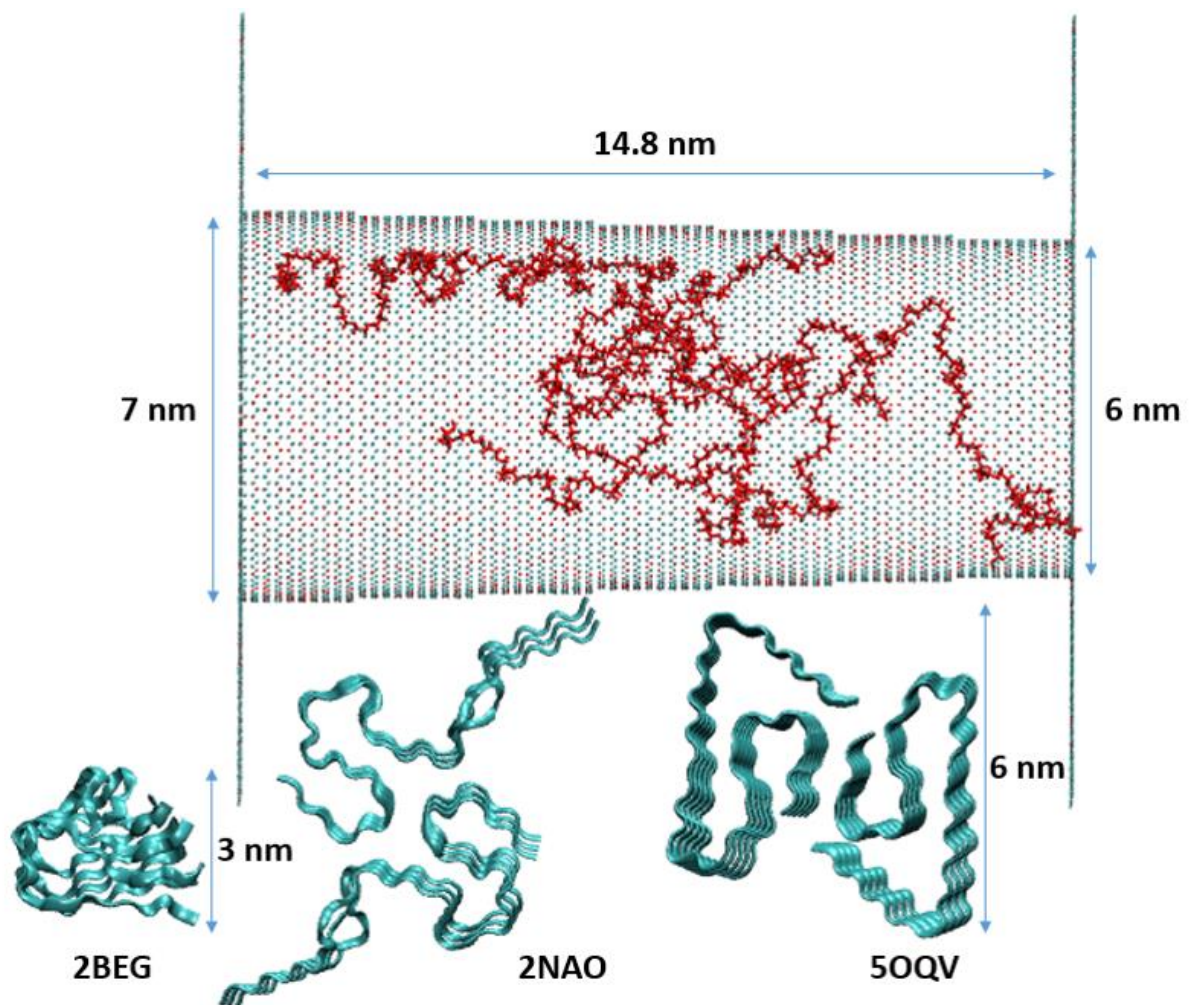
376 At this stage, several questions are still open to draw a complete description of the current
377 blockade. Indeed, we found that the dwell time is long (until second scale) and the origin of the
378 fluctuation occurring during the aggregate translocation is not fully understood. We plot in
379 Fig. S4 the relation between the standard deviation of the current blockade vs the dwell time
380 for each event. First, we can observe that the dispersion of the standard deviation increases with
381 the dwell time evidencing that the fluctuation occurs more often during the long event. Second,
382 the disparity of the standard deviation seems dependent on the incubation time and the presence
383 of pyrimethanil. Typically, for the sample incubated without pyrimethanil, it is narrow after
384 72 h incubation while in presence of pyrimethanil, it becomes narrow after 48 h. The latter
385 could be concomitant with the apparition of ordered β -sheet structure. This is in accordance
386 with a mechanism of aggregation in two steps suggested by the evolution capture rate and the
387 $\Delta I/I_0$.



388
389 **Figure 4: Average capture frequency for each experiment with (red curve) and without (black curve)**
390 **pyrimethanil.**

391 **3.3. Molecular dynamic simulation**

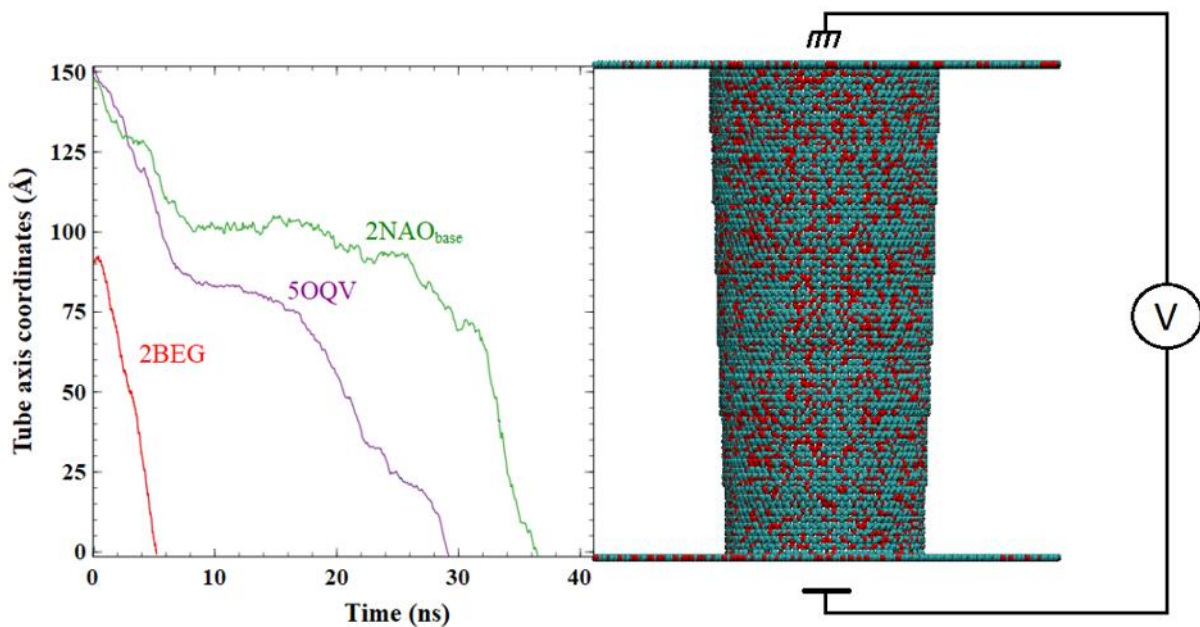
392 To provide additional insights on the A β (1-42) aggregate translocation through the nanopore,
393 we use molecular dynamic simulations. We aim to demonstrate that the interaction between the
394 nanopore and the aggregate is strongly depending on their structure. For this, we choose
395 different A β (1-42) aggregates from the PBD even if they are not strictly identical to the
396 experimental one. First, we studied the interaction between the nanopore and the A β (1-42)
397 aggregate with a single subunit structure (PDB-id 2BEG) (see Supplementary Information S5
398 and Video_S1), before generalizing this approach to the larger systems (2NAO and 5OQV PDB
399 structures, respectively) (Fig. 5). We notice that the nanopore is smaller than the experimental
400 one ($r_t = 3$ nm while $r_t = 4.7$) to keep a reasonable simulation box.



401

402 **Figure 5. Snapshots of different elements of the simulations at full scale. Top: functionalized nanopore.**
403 **Bottom from left to right: 2BEG, 2NAO and 50QV fibrils in ribbon representation.**

404 The translocation of the different fibril structures in the nanopore was investigated for two
405 voltages (0.2 V and 2 V, electric field oriented from tip to base). Under 0.2 V, we did not
406 observe A β (1-42) aggregate displacement (see Supplementary Information S5). Thus, we
407 focused our studies at a higher voltage (2 V). The translocation does not exhibit a smooth and
408 linear behaviour as shown in Figure 6. We note here that some of our attempts led to different
409 phenomena than expected. Our attempt with 2NAO (see video_S3) led to the protein adsorption
410 on the nanopore. After \sim 11 ns of stability (plateau in Figure 6), the protein splits itself in two
411 subunits with one resuming translocation while the other stays adsorbed. The 50QV protein
412 did not adopt the same behaviour, since no splitting has been observed during its translocation
413 (see video_S4 and video_S6). Note that these observations are, of course, heavily dependent on
414 the entrance's radius and of the PEG conformation.



415

416

417 **Figure 6: Position of each protein's mass centre over the axis of the nanopore. The base entrance of the**
418 **nanopore is positioned at coordinate 148.4 Å whereas tip entrance is situated at 0 Å.**

419 Once the translocation was achieved, we extracted from simulations the 1-D speed of each
 420 mass centre of protein along the nanopore axis in order to compute the average speed and to
 421 observe the different behaviours over the translocation (**Table 1**).

422 **Table 1: Translocation events of 5OQV, 2NAO and 2BEG in the different tested configurations. The**
 423 **translocation duration and the average speed of the protein were obtained through the analysis of Figure 6.**
 424 **Current blockades were computed using the analysis of the ions displacements.**

Base entrance	Translocation	Average	Number of monomer chains
	Duration	speed	(sub-units)
5OQV	27 ns	0.52 m s ⁻¹	9 (2)
2NAO	37 ns	0.41 m s ⁻¹	6 (2)
2BEG	4.6 ns	1.98 m s ⁻¹	5

425

426 When inserted from the base entrance, 5OQV and 2NAO translocation shows some sort of
 427 similarity. Both proteins adsorb around the base of the nanopore, with less PEG coating on its
 428 surface, for respectively ~8 and ~11 ns. Before and after the adsorption, we observe a quasi-
 429 linear progression inside the nanopore. 5OQV enters faster in the base part than when it travels
 430 through the tip part (speeds equal to 0.82 m s⁻¹ and 0.53 m s⁻¹ respectively, see Supplementary
 431 Information S6). This can be explained by its difficulty to leave through the smaller tip radius,
 432 its interaction with the PEG coating and the loss of its momentum while adsorbed. 2NAO shows
 433 the opposite, with a speed of 0.58 m s⁻¹ in the base part and a speed equal to 0.81 m s⁻¹ in the
 434 tip part. It has to be noted that the tip part speed is only for half the protein, as 2NAO splits
 435 during the translocation. This resulted thus from possible interactions with the nanopore wall
 436 and the PEG during the translocation of the proteins. Note that we also extracted the same

437 information for the previously described 2BEG system under a 2 V voltage which could be
438 compared to other proteins. We can thus compare speed for the last ~8 nm, after all three
439 proteins had been desorbed from the nanopore. For 5OQV (5643 atoms), half 2NAO (1881
440 atoms) and 2BEG (1870 atoms), we obtain respective speeds of 0.53 m s^{-1} , 0.81 m s^{-1} and
441 1.98 m s^{-1} . These velocities at high-voltage lead to translocation times that are still smaller than
442 experiments (27 ns, 37 ns and 4.6 ns, respectively).

443 Even if the molecular dynamic simulation was not performed under strictly the same
444 condition as the experiments. The results allow providing elements to better understand the long
445 dwell time. Indeed, as described in Supplementary Information, at low voltage (0.2 V), the
446 interaction of the protein with the functionalized nanopore remained so high that no net
447 displacement of the A β (1-42) aggregate was observed. The adhesion of the protein, under the
448 combined effect of a longer nanopore and a lower induced voltage, seems thus to have a crucial
449 role in the amyloid dwell time as suggested by the different simulation conditions. In addition,
450 the simulation highlights a slowdown of the A β (1-42) aggregate when it reaches the PEG chain.
451 Thus, it is plausible that similar scenario occurs inside conical nanopore, explaining long dwell
452 time experimentally observed. According to theoretical conclusion, we could speculate that the
453 small aggregate does not enter in interaction with the PEG and thus translocate faster (no event
454 is observed), conversely the aggregate that reaches a critical size promoting a strong interaction
455 with PEG is detected.

456 **4. Conclusions**

457 Our work aimed to investigate the effect of pyrimethanil on A β (1-42) aggregation. Using
458 conical nanopores functionalized with PEG, we successfully detected A β (1-42) aggregates at
459 different time during the lag phase where the oligomer are often too small to be detected by
460 other techniques. Regardless the incubation time and the presence of pyrimethanil, long large
461 current (>0.7) blockades are observed. Using an analytical model only based on geometrical

462 consideration, we could assign the detectable events to the A β (1-42) aggregates with a size
463 close to that of the sensing pore diameter. The molecular dynamic simulations confirm that the
464 A β (1-42) aggregates can translocate through the nanopore but interact with PEG explaining the
465 long dwell time (hundreds ms scale) experimentally recorded. They also evidence amyloid/pore
466 interaction in the zone without PEG coating confirming its importance. Such interaction let
467 think splitting phenomena of the dimer structure occurs. The A β (1-42) aggregates interaction
468 with PEG and the uncoated zones will make the experimentalist to optimize the antifouling
469 functionalization.

470 Generally speaking, by combining conventional techniques and single nanopore-based
471 technology, our work confirms pyrimethanil as an enhancer of A β (1-42) assembly during the
472 lag phase of fibril formation. More interestingly, the nanopore approach evidences that the
473 pyrimethanil induce an aggregation of A β (1-42) mechanism in two steps including the
474 reorganization after 48 h at the beginning of the elongation phase. This supports the emerging
475 theory that the fibril formation involved numerous phases of dissociation, conversion and
476 fragmentation. The original result here is that such phenomena can occur quickly in presence
477 of pyrimethanil. This opens numerous questions about the health impact of certain chemicals
478 used in agriculture.

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489

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