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1 Taylor Dispersion Analysis and Atomic Force
2 Microscopy provide quantitative insight on the
3 aggregation kinetics of A β (1-40)/A β (1-42) amyloid
4 peptide mixtures

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17 **ABSTRACT**

18 Aggregation of amyloid β peptides is known to be one of the main processes responsible for
19 Alzheimer's disease. The resulting dementia is believed to be due in part to the formation of
20 potentially toxic oligomers. However, the study of such intermediates and the understanding of
21 how they form is very challenging because they are heterogeneous and transient in nature.
22 Unfortunately, few techniques can quantify, in real time, the proportion and the size of the different
23 soluble species during the aggregation process. In a previous work (Deleanu *et al.* Anal. Chem.
24 2021), we showed the potential of Taylor dispersion analysis (TDA) in amyloid speciation during
25 the aggregation process of $A\beta(1-40)$ and $A\beta(1-42)$. The current work aims at exploring in detail
26 the aggregation of amyloid $A\beta(1-40):A\beta(1-42)$ peptide mixtures with different proportions of each
27 peptide (1:0, 3:1, 1:1, 1:3 and 0:1), using TDA and atomic force microscopy (AFM). TDA allowed
28 for monitoring the kinetics of the amyloid assembly and quantifying the transient intermediates.
29 Complementarily, AFM allowed the formation of insoluble fibrils to be visualized. Together, the
30 two techniques enabled to study the influence of the peptide ratios on the kinetics and the formation
31 of potentially toxic oligomeric species.

32

33 **KEYWORDS.** Taylor dispersion analysis; AFM; peptide aggregation; oligomers; amyloid beta
34 peptides; diffusion coefficient; hydrodynamic radius.

35

36

37 **Introduction**

38 Alzheimer's disease (AD) is the most common primary dementia. It usually presents a
39 progressive course and characteristically affects different cognitive and behavioral functions.
40 Perhaps the cardinal, most frequently observed symptom of disease onset is memory loss¹, which
41 results from initial lesions in the hippocampus (which lies in the medial temporal lobes of the brain
42 and is responsible for long-term memory) further extending to the rest of the brain. At later stages,
43 the degeneration of other cognitive and behavioral areas is observed which will clearly indicate
44 the type of dementia.

45 The brains of Alzheimer's patients present a series of characteristic hallmarks. First,
46 neurofibrillary tangles composed of hyperphosphorylated tau protein are observed in neurons.
47 Then, extracellular fibrillary structures called neuritic (or senile) plaques, that are due to the
48 deposition of amyloid β peptides ($A\beta$), are observed². In the 1990's, it was believed that the senile
49 plaques were the most pathogenic forms of the $A\beta^{3,4}$, which resulted from the self-assembly of the
50 two major amyloid peptides $A\beta(1-40)$ and $A\beta(1-42)$. During this self-assembly, species are formed
51 with evolving morphology and size from oligomers, to protofibrils and finally to fibrils and
52 plaques, through a highly complicated process.

53 More recent studies suggested that the main factor of AD pathogenesis was the formation of
54 soluble oligomers of $A\beta$, which are believed to be more toxic than plaques because they are able
55 to spread across neuronal tissue and bind to membrane receptors, including the prion protein,
56 promoting neurotoxicity and synaptic loss⁵⁻⁷. However, in contrast to fibrils, which are highly
57 stable and can be observed by microscopy, the soluble oligomers are more difficult to detect and
58 to study in real time⁸, since they are metastable, transient⁸ and highly polydisperse in size. Studies
59 have shown that $A\beta(1-40)$ does not quantitatively form small oligomers during the aggregation

60 process but rather goes from monomers to fibrils following a direct pathway^{9,10}. On the other hand,
61 A β (1-42) goes through the formation of intermediate species with diverse sizes and shapes^{11,12}.
62 Although these two amyloid peptides coexist *in vivo*^{13,14}, most of the *in vitro* studies on A β were
63 focused on the study of pure peptide solutions and only a small proportion of the vast AD literature
64 was dedicated to mixtures of these peptides¹⁵⁻¹⁸. Many of the studies dealing with the mixtures
65 were directed toward the kinetics of the aggregation process and more particularly the study of the
66 amyloid fibers^{15-17,19-21} and very few toward the oligomeric structures¹⁸. The aggregation process
67 in such mixtures was already studied by Thioflavin T fluorescence^{17,21}, sedimentation¹⁵, atomic
68 force microscopy (AFM)^{16,19}, nuclear magnetic resonance (NMR)^{22,23} or electron paramagnetic
69 resonance¹⁸ to name few methods. The results from these studies indicated that both A β (1-40) and
70 A β (1-42) interact during the aggregation process, with cross seeding between peptides. However,
71 some authors claimed that the fibrils are homomolecular¹⁷ while others stated that
72 heteromolecular¹⁸ fibrils are obtained. In all cases, it was observed that the presence of A β (1-42)
73 accelerated the aggregation of A β (1-40) and vice versa. To our knowledge, an in-depth study on
74 the species present during the early stages of the aggregation of amyloid peptide mixtures does not
75 exist to date. For that reason, and to help develop drug candidates targeting the toxic oligomers,
76 new analytical methodologies are required to monitor and size the different species in real time.

77 In this context, Taylor dispersion analysis²⁴⁻²⁶ (TDA) appears as a very promising alternative
78 analytical method. In our previous report²⁷, we showed the ability of TDA to follow the
79 aggregation process of amyloid peptides, using an extensive data treatment that revealed a
80 complete picture of the aggregation process and allowed to size the transient structures. As
81 described elsewhere, TDA allows to determine the molecular diffusion coefficient, D , and
82 hydrodynamic radius, R_h , of a solute, including for mixtures, without any bias in size as compared

83 to other sizing methods²⁸, since the small and the large solutes contribute proportionally to their
84 mass abundance in the mixture²⁹. The use of TDA in the field of protein aggregation presents
85 several advantages³⁰⁻³³. It offers low sample consumption (less than 1 μ L for the whole aggregation
86 process), short analysis time allowing a high number of sampling points and real time monitoring
87 of the aggregation, a wide range of sizing (from angstrom to sub-micron) allowing to size the
88 monomers, oligomers and higher size soluble prefibrillar structures, together with a direct analysis
89 without any sample pretreatment or filtration³⁴⁻³⁶.

90 In this work, we used TDA to study peptide mixtures of A β (1-40) and A β (1-42) with the aim of
91 revealing the size of the transient structures formed during the aggregation process. The results for
92 selected time points were compared with those obtained by AFM to correlate the observations
93 from these two independent techniques. AFM also allowed to study non-soluble fibrils, which
94 cannot be sized or directly observed by TDA.

95 **Materials and methods**

96 **Materials**

97 Amyloid beta (1-40) (denoted A β (1-40) in this work) was prepared by fast conventional solid
98 phase peptide synthesis (SPPS) using a *Fmoc* orthogonal strategy as described elsewhere²⁷.
99 Amyloid beta (1-42) (A β (1-42), batch number 100002591, >95%) was purchased from Bachem
100 (Bubendorf, Switzerland). Sodium dihydrogen phosphate, tris(hydroxymethyl)aminomethane,
101 hydrochloric acid fuming 37%, sodium chloride and sodium hydroxide were purchased from
102 Sigma Aldrich (France). The ultrapure water used for all buffers was prepared with a MilliQ
103 system from Millipore (France).

104 **Peptide pretreatment**

105 Both A β (1-40) and A β (1-42) were first pretreated independently as described elsewhere^{37, 38}.
106 Briefly, A β (1-40) and A β (1-42) were dissolved in a 0.10 % (m/v) and 0.16 % (m/v) NH₄OH
107 aqueous solution respectively to reach a final peptide concentration of 2 mg/mL. The peptide
108 solutions were then incubated at room temperature for 10 min, separated into several aliquots and
109 freeze-dried. The aliquot volume was calculated in order to obtain 10 nmol of peptide in each
110 Eppendorf tube. The lyophilized peptide aliquots were stored at -20 °C until further use.

111 **Peptide aggregation study by Taylor dispersion analysis**

112 Briefly, and as described thoroughly elsewhere^{25, 29, 30, 39}, Taylor dispersion analysis allows for the
113 determination of the molecular diffusion coefficient of a solute which can be obtained from the
114 band broadening resulting from the combination of the Poiseuille parabolic flow and the molecular
115 diffusion by quantifying the temporal variance (σ^2) of the elution profile. For that, a Gaussian
116 function is used to fit the experimental elution peak allowing to obtain the peak variance σ^2 and
117 thus calculate the molecular diffusion coefficient, D , and consequently the hydrodynamic radius,
118 R_h . When more than one size populations are present, a sum of Gaussian functions can be used to
119 fit the experimental trace, or Constrained Regularized Linear Inversion (CRLI)⁴⁰ can be used to
120 get the size distribution. For more details, the reader may refer to the supporting information for
121 the theoretical aspects, equations and more details on the data processing.

122 TDA was performed on an Agilent 7100 (Waldbronn, Germany) capillary electrophoresis system
123 using bare fused silica capillaries (Polymicro technologies, USA) having 40 cm \times 50 μ m i.d.
124 dimensions and a detection window at 31.5 cm. New capillaries were conditioned with the
125 following flushes: 1 M NaOH for 30 min; ultrapure water for 30 min. Between each analysis,
126 capillaries were rinsed with 20 mM phosphate buffer, pH 7.4 (2 min). Samples were injected
127 hydrodynamically on the inlet end of the capillary (44 mbar, 3 s, injected volume was about 7 nL

128 corresponding to 1% of the capillary volume to the detection point). Experiments were performed
129 using a mobilization pressure of 100 mbar. The temperature of the capillary cartridge was set at
130 37 °C. The vial carousel was thermostated using an external circulating water bath 600F from
131 Julabo (Germany). The solutes were monitored by UV absorbance at 191 nm. The mobile phase
132 was a 20 mM, pH 7.4 phosphate buffer (viscosity at 37 °C is 0.7×10^{-4} Pa.s). To prepare the
133 mixtures, freeze-dried mixed peptide aliquots were prepared so that the final sample would contain
134 13 nmol of total peptide except for the pure samples where the amount of peptide was of 10 nmol.
135 First, each of the required stock aliquots (see previous section) were dissolved in 100 μ L of 0.16%
136 (m/v) NH_4OH to avoid aggregation during this step, and appropriate volumes were used to obtain
137 the desired mixtures. The final aliquots were immediately subjected to freeze-drying and then
138 stored at -20°C until further use. The resulting peptide powders were dissolved in 20 mM
139 phosphate buffer at pH 7.4 to reach a final total peptide concentration of 133 μM : *i*) 100 % $\text{A}\beta(1-$
140 40) contained 10 nmol of $\text{A}\beta(1-40)$ dissolved in 75 μL of buffer; *ii*) 75 % $\text{A}\beta(1-40)$ and 25 %
141 $\text{A}\beta(1-42)$ mixture contained 10 nmol of $\text{A}\beta(1-40)$ and 3.33 nmol of $\text{A}\beta(1-42)$ dissolved in 100 μL
142 of buffer; *iii*) 50 % $\text{A}\beta(1-40)$ and 50 % $\text{A}\beta(1-42)$ mixture contained 6.67 nmol of each peptide and
143 was dissolved in 100 μL of buffer; *iv*) 25 % $\text{A}\beta(1-40)$ and 75 % $\text{A}\beta(1-42)$ mixture contained 3.33
144 nmol of $\text{A}\beta(1-40)$ and 10 nmol of $\text{A}\beta(1-42)$ dissolved in 100 μL of buffer and finally *v*) 0 % $\text{A}\beta(1-$
145 40) contained 10 nmol of $\text{A}\beta(1-42)$ dissolved in 75 μL of buffer. After dissolution, the mixtures
146 were immediately transferred to a capillary electrophoresis vial and incubated at 37°C in the
147 capillary electrophoresis instrument carousel. Aggregation was monitored by injecting the sample
148 ($V_{inj} \approx 7$ nL) every 7 min in the case of pure $\text{A}\beta(1-42)$ and the $\text{A}\beta(1-40)$: $\text{A}\beta(1-42)$ 1:3 mixture (25
149 % $\text{A}\beta(1-40)$), while it was injected every 20 min for the $\text{A}\beta(1-40)$: $\text{A}\beta(1-42)$ 1:1 mixture (50 %
150 $\text{A}\beta(1-40)$) and every 30 min in the case of pure $\text{A}\beta(1-40)$ and the $\text{A}\beta(1-40)$: $\text{A}\beta(1-42)$ 3:1 mixture

151 (75 % A β (1-40)). During the monitoring of the aggregation process, each sample was injected for
152 100 to 125 TDA runs, corresponding to a total injected sample volume between 700 nL and 875
153 nL. To avoid sample evaporation, the vial cap was changed three times a day. The taylorgrams
154 were recorded with the Agilent Chemstation software, then exported to Microsoft Excel for
155 subsequent data processing. In general, the obtained elution profiles were not Gaussian, meaning
156 that the sample was polydisperse in size. All taylorgrams were fitted on the basis of the right-side
157 elution profile (*i.e.* $t > t_0$, with t_0 the peak time) to remove the spikes present on the left side as
158 described elsewhere²⁷.

159 **Atomic force microscopy (AFM)**

160 For atomic force microscopy characterisation, 5 μ L aliquots of the peptide solutions collected at
161 different aggregation times were dried on silicon substrates freshly cleaned with piranha solution,
162 before gently rinsing with ultrapure water and drying in a flow of nitrogen gas. An Agilent 5500
163 AFM system with MSNL-F cantilevers ($f = 110$ - 120 kHz, $k = 0.6$ N/m, average tip radius of 2-12
164 nm) was used for topographical imaging in intermittent contact mode. The AFM topography
165 images were levelled, line-corrected and analysed using Gwyddion⁴¹, a free and open-source SPM
166 (scanning probe microscopy) data visualization and analysis program. Maxima analysis was
167 performed using ImageJ⁴².

168 **Results and discussion**

169 **Taylor Dispersion Analysis and data processing**

170 One main objective of this work is to show the influence of the relative proportion of A β (1-40)
171 with respect to A β (1-42) on the aggregation process. The aggregation of the peptides in the
172 different mixtures was followed at 37°C. Figure 1 shows the taylorgrams recorded at selected

173 incubation times for A β (1-40), A β (1-40):A β (1-42) mixtures (with molar ratio of A β (1-40) in the
174 mixture of 25%, 50% and 75% corresponding to 3:1, 1:1 and 1:3 mixtures), and A β (1-42). The
175 total peptide concentration in each solution was set at 133.3 μ M. All experimental taylorgrams,
176 for all incubations times t_{ag} , are shown in Figures SI.1 to SI.5. Figure 1 allows for a visual
177 comparison of the aggregation kinetics between the different amyloid peptide ratios. The
178 absorbance decrease of the elution profile with t_{ag} , which is due to the decrease in concentration
179 of the soluble species, was faster when increasing the A β (1-42) content in the mixture, as
180 previously observed for pure peptide solutions²⁷.

181 In order to elucidate the aggregation process in these solutions, an extensive data treatment was
182 realized on all the obtained taylorgrams. As already observed in our previous report²⁷, sharp peaks
183 or spikes, sometimes appear on the left side of the elution peak because of the presence of large
184 fibrils in suspension which are out of the Taylor regime^{43, 44} and/or due to specific hydrodynamic
185 behavior for suspended large aggregates⁴⁵. The presence of these spikes imposes a data treatment
186 on the right side of the elution profile. Two different approaches were used to treat the
187 experimental elution profiles. First, the fitting with a finite number of Gaussian functions ($n = 4$ in
188 this work), leads to the classification of the obtained size populations into four categories: (i) small
189 unidentified molecules ($R_h < 0.9$ nm), (ii) monomers and small oligomers (0.9 nm $< R_h < 5$ nm),
190 (iii) higher mass oligomers (5 nm $< R_h < 50$ nm) and finally (iv) soluble protofibrils (50 nm $< R_h$
191 < 300 nm). The second approach is based on the Constrained Regularized Linear Inversion (CRLI)
192 which aims at finding the probability density function $P_D(D)$ that fits the taylorgram without any
193 hypothesis on the number of populations⁴⁰.

194 **Aggregation process of pure and mixed solutions of A β peptides by TDA and AFM**

195 In the case of A β (1-40) alone, the first sign of spikes (fibrils) appears at $t_{ag} \sim 12$ h (Figure SI.1).
196 However, the spike intensity remained relatively low and did not increase drastically over the
197 whole aggregation process (see gray data points in Figure 2). In parallel, the monomer peak
198 intensity remained constant until $t_{ag} \sim 20$ h (“lag phase”), and then decreased rapidly to reach a
199 lower plateau after $t_{ag} \sim 48$ h (red squares in Figure 2). This suggests an initial slow aggregation
200 step followed by a rapid fibrillization catalyzed by the formation of large aggregates (seeds) that
201 do not enter the capillary upon injection⁴⁶, in accordance with our previous report where another
202 concentration (100 μ M) of A β (1-40) was used²⁷.

203 On the contrary, A β (1-42) alone did not show any lag phase and a fast decrease of this population
204 was observed with a complete disappearance after less than 2 h (red squares in Figure 2).
205 Meanwhile, when mixed together, A β (1-42) seemed to increase the kinetics of aggregation, with
206 the monomer population decreasing after 15 h, 8 h and 6 h for the 75%, 50% and 25% A β (1-40)
207 mixtures respectively, without any visible lag phase (red squares in Figure 2).

208 With the aim of verifying hypotheses formulated from TDA, we also performed AFM imaging for
209 key times of the fibrillization process. The findings by TDA correlated well with AFM
210 observations for A β (1-40) alone (Figure 3), which showed the number of fibrils rise only at $t_{ag} =$
211 28.10 h. In AFM images, the substrate remained covered with spherical objects in the 10 nm
212 diameter range (including eventual tip convolution effects) that can be attributed to monomers and
213 oligomers. The coverage by these species only decreased significantly, exposing portions of the
214 bare substrate, for $t_{ag} = 28.10$ h, which demonstrates the consumption of these objects by the
215 fibrillization process.

216 In the case of A β (1-42) alone, the kinetics of aggregation were much faster than that of A β (1-40),
217 as demonstrated by the taylorgrams (Figure 1) and the monomer consumption (red squares in
218 Figure 2). The first spikes were observed only after $t_{ag} \sim 10$ min (Figure SI 5), in agreement with
219 AFM showing fibrils for the shortest incubation times, and a complete disappearance of the soluble
220 peptide species was observed after $t_{ag} \sim 7$ h (Figure 3). Similarly, only few monomeric/oligomeric
221 species remained visible after 48 min in AFM images, which showed from this time onward mainly
222 the substrate and fibrils. The decrease in the number of fibrils observed by microscopy for the
223 longest incubation times is likely due to the tangling of fibrils and compaction of these aggregates,
224 that have less affinity for the substrate and are more easily removed during the rinsing and drying
225 steps, as observed for other amyloid systems⁴⁷.

226 When mixed together, aggregation/fibrillization observed by both TDA and AFM accelerated with
227 the proportion of A β (1-42). In the case of the 75% A β (1-40) mixture, the first spikes in TDA
228 appeared after $t_{ag} \sim 6.5$ h (Figure SI.2), while they were observed after only ~ 1.5 h and ~ 1 h for the
229 50 % and 25 % A β (1-40) mixtures respectively (Figures SI.3 and SI.4 respectively). In the case of
230 the mixture with the highest amount of A β (1-40), the spikes intensity increased with time and
231 remained visible even after 60 h of aggregation suggesting that the formed fibrillary structures are
232 smaller in size than those formed with A β (1-40) alone, and can enter more easily in the capillary
233 during the injection step. These observations were also conveyed in the AFM images, with fibers
234 appearing increasingly early during the aggregation process, and the spherical objects attributed to
235 monomers and oligomers being consumed faster upon raising the proportion of A β (1-42). Indeed,
236 these small species remain predominant on the substrates up to 28.10, 8.15, 3.05 and 0.27 h
237 respectively for 0, 25, 75 and 100% A β (1-42).

238 As mentioned earlier, the Taylorgrams were treated by fitting a sum of Gaussian functions to
239 extract the abundance of size populations present under each elution peak during the aggregation
240 process. Figure 4 shows the hydrodynamic radii of the populations in the studied mixtures as a
241 function of incubation time, while Table 1 shows the average hydrodynamic radii values for the
242 size populations and the different peptide mixtures. As can be seen in Figure 4, the size of each
243 population was relatively constant during the aggregation process. The population called
244 ‘monomer’ corresponds to the monomers and small oligomers with an average size of about 2 nm
245 in all mixtures. However, the statistical analysis showed that the monomer populations in A β (1-
246 40) and in the 25 % A β (1-40) mixture were significantly smaller at a 95% confidence level than
247 for the other three mixtures, based on the average hydrodynamic radii obtained over the whole
248 aggregation study for each mixture (see Table 2 and Figure SI.6). Further, A β (1-40) alone showed
249 the lowest ‘monomer’ population size average value of 1.81 ± 0.11 nm, while the 25 % A β (1-40)
250 mixture showed the highest average value of 2.42 ± 0.20 nm. While the other three mixtures, 75
251 % A β (1-40) mixture, 50% A β (1-40) mixture and A β (1-42) alone were not statistically different
252 (see R_h values in Table 2). These results suggest that when the mixture contained an excess of
253 A β (1-42) as compared to A β (1-40), the formed oligomers were larger in size than those obtained
254 for A β (1-42) alone and for the mixtures with a higher amount of A β (1-40). To explain this
255 observation, the following hypothesis is proposed. First, it was already shown that A β (1-40)
256 mainly forms fibrils directly from the monomeric state without passing through intermediate
257 species^{9,10}. The presence of the first fibrils of A β (1-40) catalyzed by the presence of A β (1-42) may
258 play the role of seeds for the A β (1-42) peptide²¹, and thus, oligomers with a higher molar mass are
259 formed. When the amount of A β (1-42) decreases, the aggregation process tends to follow the
260 pathway characteristic of A β (1-40) (direct formation of fibrils), and thus, the smallest oligomeric

261 species tend to remain monomeric with a lower average size. For AFM, in the presence of
 262 biomolecules, the lateral dimensions are often affected by tip convolution, leading to unpredictable
 263 broadening of surface features^{48, 49}. We therefore measured the average heights of the various
 264 objects, which gave a good estimation of radii. The results obtained on the different mixtures of
 265 the radii (at times enabling to measure isolated objects) for small spherical objects attributed to
 266 monomers/oligomers, and the derived half-widths for the fibers are gathered in Table 2. The radii
 267 of monomers/oligomers are in good agreement between TDA and AFM, although values measured
 268 for monomers, oligomers and fibers with AFM are not significantly different between ratios.

269 **Table 2:** Average hydrodynamic radius in nm obtained by TDA over the whole aggregation process
 270 of each mixture. Average heights in nm obtained by AFM on the small spherical objects and on
 271 the fibrils for the different A β mixtures and at different aggregation times.

	TDA	AFM			
		Small spherical objects		Fibrils	
% A β (1-40) in A β (1-40):A β (1-42) mixtures	Average R_h (nm) (n = number of points)	Radii \pm SD (nm)	t_{ag} (h)	Half-width \pm SD (nm)	t_{ag} (h)
0	1.96 \pm 0.10 (n = 121)	2.15 \pm 0.40	1.47	2.89 \pm 0.70	0.8
25	2.42 \pm 0.20 (n = 77)	2.22 \pm 0.44	8.02	2.78 \pm 0.72	27.83
50	2.00 \pm 0.25 (n = 42)	N.A.	N.A.	N.A.	N.A.
75	1.98 \pm 0.24 (n = 77)	2.32 \pm 0.44	12.23	3.02 \pm 1.17	27.97
100	1.81 \pm 0.11 (n = 53)	2.43 \pm 0.60	28.10	3.53 \pm 0.93	28.10

272

273 As for the higher mass oligomer population, the size remained relatively constant throughout the
274 aggregation process within a given mixture. However, it was observed that with an increase in the
275 A β (1-40) proportion, the average size for the oligomer population was higher than in the case of
276 an excess of A β (1-42) (~18 nm as compared to ~9 nm) (Figure SI.6). Moreover, a sudden increase
277 of the oligomer R_h from about 10 nm to ~30 nm can be observed when the “monomer” population
278 decreased in area (Figure 2 for the size and Figure 4 for the area) after ~2h for A β (1-42), 24 h for
279 the 75% A β (1-40) mixture and 32 h for A β (1-40). This effect was not observed for the 50% and
280 25% A β (1-40) mixtures. Further, Figure 2 shows that this oligomeric population reaches a
281 maximum in concentration at around 30 min for the A β (1-42) sample, while this maximum is
282 shifted to higher times with the decrease in A β (1-42) proportion (1.3 h, 1.6 h, 5.5 h and 16 h for
283 25%, 50%, 75% and 100 % A β (1-40) mixtures, respectively). Recent studies suggested that
284 amyloid peptides can undergo liquid-liquid phase separation (LLPS) before the formation of
285 amyloid fibrils⁵⁰⁻⁵². The “high mass oligomers” population with R_h ranging from 5 to 50 nm found
286 in this work, might correspond to high-density protein condensates. The size increase of the species
287 over time can be explained by Ostwald ripening.

288 Regarding the protofibril population, the R_h values varied between 80 and 140 nm, for all the
289 mixtures independently of the peptide proportions (Figure 4).

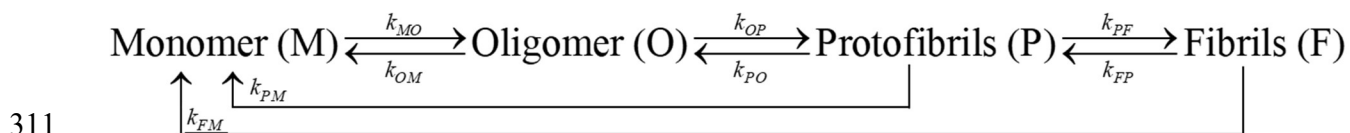
290 **Hydrodynamic radius distributions by CRLI**

291 To get a deeper insight into the evolution of the species during the aggregation process, CRLI
292 analysis^{27, 40} was applied on the right part of the taylorgrams (i.e. for $t > t_0$). Figure 5 shows the
293 hydrodynamic radii distributions obtained by CRLI on TDA runs for selected aggregation times
294 for all studied samples, while Figures SI.7 to SI.11 show the distributions over the whole

295 aggregation process for the studied samples. From these distributions, one can note that for A β (1-
 296 40) alone, only the monomer and small oligomer populations were observed throughout the
 297 aggregation process without the notable appearance of intermediate species. On the contrary,
 298 A β (1-42) aggregation led to intermediate oligomers having an average size around 10 nm
 299 accompanied with a broadening and disappearance of the monomeric population in accordance
 300 with our previous report²⁷. When mixed together, the presence of intermediate species became
 301 more noticeable with the increase in A β (1-42) proportion. These results provide an explanation to
 302 the increase in A β mixtures toxicity with an increase in the A β (1-42)/A β (1-40) ratio^{19, 23, 53},
 303 supporting the notion that this toxicity correlates with the amount of intermediate oligomeric
 304 species.

305 Kinetic analysis of the aggregation process

306 One of the major advantages of using TDA combined with advanced data treatment is the
 307 possibility to get the size distributions of the different populations with high throughput during the
 308 whole aggregation process. These distributions allow gaining insight on the aggregation
 309 mechanism, by modelling the data based on the chain of association and dissociation reactions
 310 shown in Figure 6⁵⁴.



312 **Figure 6.** Scheme of the association and dissociation reactions of amyloid species used to model
 313 the aggregation process (adapted from⁵⁴). The rate constant for each reaction is indicated close to
 314 the respective reaction arrow.

315 The forward and backward reactions going from the monomer (M) population to the oligomers
 316 (O), the protofibrils (P), and finally the fibrils (F), as shown in Figure 6, are modelled by the
 317 following set of equations:

$$318 \quad \frac{dM(t)}{dt} = -k_{MO}M(t) + k_{OM}O(t) + k_{PM}P(t) + k_{FM}F(t) \quad (1)$$

$$319 \quad \frac{dO(t)}{dt} = -(k_{OP} + k_{OM})O(t) + k_{MO}M(t) \quad (2)$$

$$320 \quad \frac{dP(t)}{dt} = -(k_{PF} + k_{OM})P(t) + k_{OP}O(t) \quad (3)$$

$$321 \quad \frac{dF(t)}{dt} = k_{PF}P(t) - k_{FM}F(t) \quad (4)$$

322 where, M(t), O(t), P(t) and F(t) are the concentrations of the monomers, oligomers, protofibrils
 323 and fibrils respectively. k_{MO} is the rate for the reaction transforming monomers into oligomers;
 324 similar notations are used for the other rate constants. Note that TDA does not allow the fibrils
 325 population to be directly measured, so only equations (1-3) were used. We fit Eqs. (1-3) to the
 326 temporal evolution of the population distributions obtained by TDA, by assuming that the
 327 (integrated) absorbance signal for each species is proportional to its concentration, and that the
 328 same proportionality constant applies to all species. Equation (4) was not used since TDA does
 329 not allow the direct quantification of the fibril concentrations. The fit is performed using custom
 330 software based on the Scipy package⁵⁵, performing a least square minimization of the set of rate
 331 constants, solving numerically Eqs. (1-3) at each iteration. The resulting fitting curves are shown
 332 as dot-dashed lines in Figure 2 (see also Figures SI.12 to SI.16), while the fitting parameters are
 333 reported in Table 1. Note that for most samples the monomer population asymptotically tends for
 334 large t to a non-zero concentration value, due to the dissociation of the higher-order species. The
 335 100 % A β (1-40) could not be fitted entirely, because of the presence of a lag phase at early times.

336 However, data for this sample could be fitted by excluding the monomer population data points
337 for $t < \sim 20$ h. For all other sample mixtures, the fit allowed to extract the kinetics rate constants
338 reported in Table 1. From these values, it can be deduced that the reaction rates tend to decrease
339 with increasing amounts of A β (1-40) in the mixture. For the 0% A β (1-40) sample, the forward
340 reactions were dominant as compared to the backward ones, suggesting that the aggregation is
341 close to an irreversible reaction rather than to an equilibrium one. When a small amount of A β (1-
342 40) is added (25%), the reaction rates of forward and backward reactions become similar. These
343 results can explain the higher toxicity of this A β ratio observed in the literature¹⁹. Indeed, the
344 backward reactions correspond to the dissociation of higher order species into monomers or low
345 molar mass oligomers, which are known to be toxic. To our knowledge, this is the first time that
346 kinetic rates of the aggregation mechanism of A β peptides could be determined by measuring
347 directly the monomer and oligomer distributions. When combined with models proposed in the
348 literature, mainly based on measurements of the time-dependent aggregate mass^{54, 56, 57} (e.g. by
349 ThT fluorescence assay), or numerical simulations⁵⁸, TDA data as those presented here will help
350 reaching a comprehensive understanding of the aggregation process of these amyloid species,
351 potentially contributing to assess the effectiveness of drugs targeting the toxic oligomeric species.

352 **Conclusion**

353 This work demonstrated the interest of Taylor dispersion analysis to assist the study of complex
354 amyloid peptide mixtures and shed more light on the aggregation process in these systems. The
355 comparison of the results obtained in parallel by TDA and AFM showed the complementarity of
356 the two techniques, where TDA is able to quantify and size of small objects while AFM can size
357 the fibrillary structures not accessible by TDA. In addition, the results confirmed that the kinetics

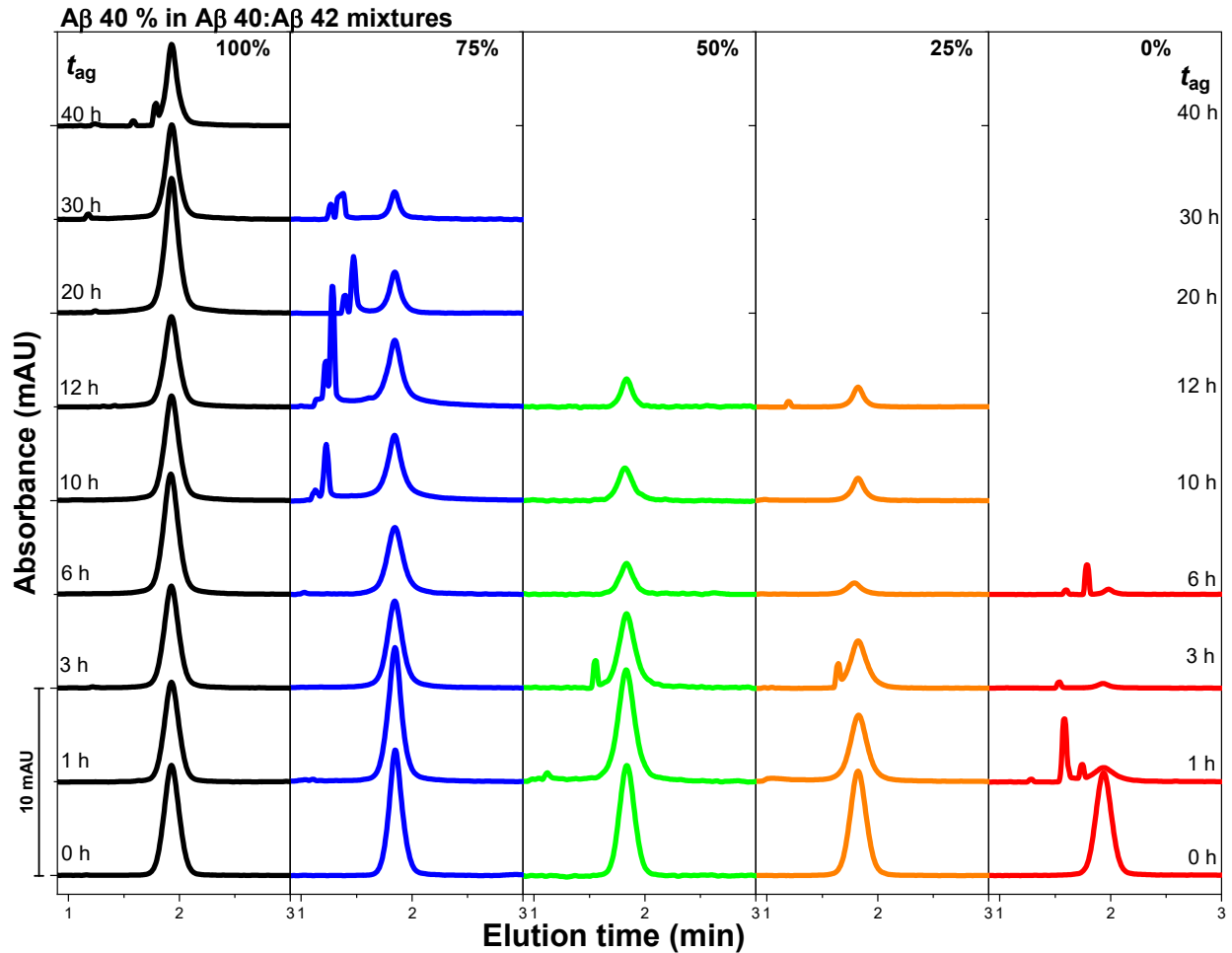
358 of aggregation strongly depend on the nature of amyloid-forming peptides and their environment.
359 Under our working conditions, the more amyloid-prone A β (1-42) aggregates more rapidly
360 (minutes scale) compared to A β (1-40), which aggregates in about 24h, in agreement with
361 previously published works^{27, 59}. When mixing together the two species, the aggregation rate was
362 highly influenced by the ratio of A β (1-40):A β (1-42). Indeed, A β (1-42) was found to accelerate the
363 aggregation rate of A β (1-40), probably by a cross-seeding mechanism. For example, the
364 disappearance of the monomeric species decreased from 48 h in the case of 100 % A β (1-40) to 12
365 h when 25 % of A β (1-42) were present in the sample.

366 In addition to the clear observation on the interplay between both A β peptides during the
367 aggregation process of mixtures and the influence of the A β ratio on the aggregation rate, this work
368 shows that this ratio modulates the formation of potentially toxic oligomers. In fact, when the
369 peptides were mixed together, intermediate oligomeric species were observed and tended to
370 increase in proportion upon increasing the A β (1-42) content. Modifying the A β ratio changed the
371 onset of the oligomeric species appearance and monomeric species disappearance (monomers and
372 small oligomers R_n lower than 5 nm), as well as the aggregation mechanism (direct formation of
373 fibrils or formation of intermediate species). These results support the importance of understanding
374 the mechanism of the aggregation process in the case of A β mixtures (in better accordance with
375 the *in vivo* conditions), to better direct research toward an AD therapy able to inhibit the formation
376 of intermediate species depending on the A β ratio. In this respect, TDA was shown to be a
377 straightforward method able to give with unprecedented detail new insight on the size and
378 distribution of the species formed during the aggregation process. Finally, the combination of TDA
379 with extensive data processing and highly resolved efficient methods such as AFM paves the way
380 for building a comprehensive picture of the speciation and growth processes, as illustrated here for

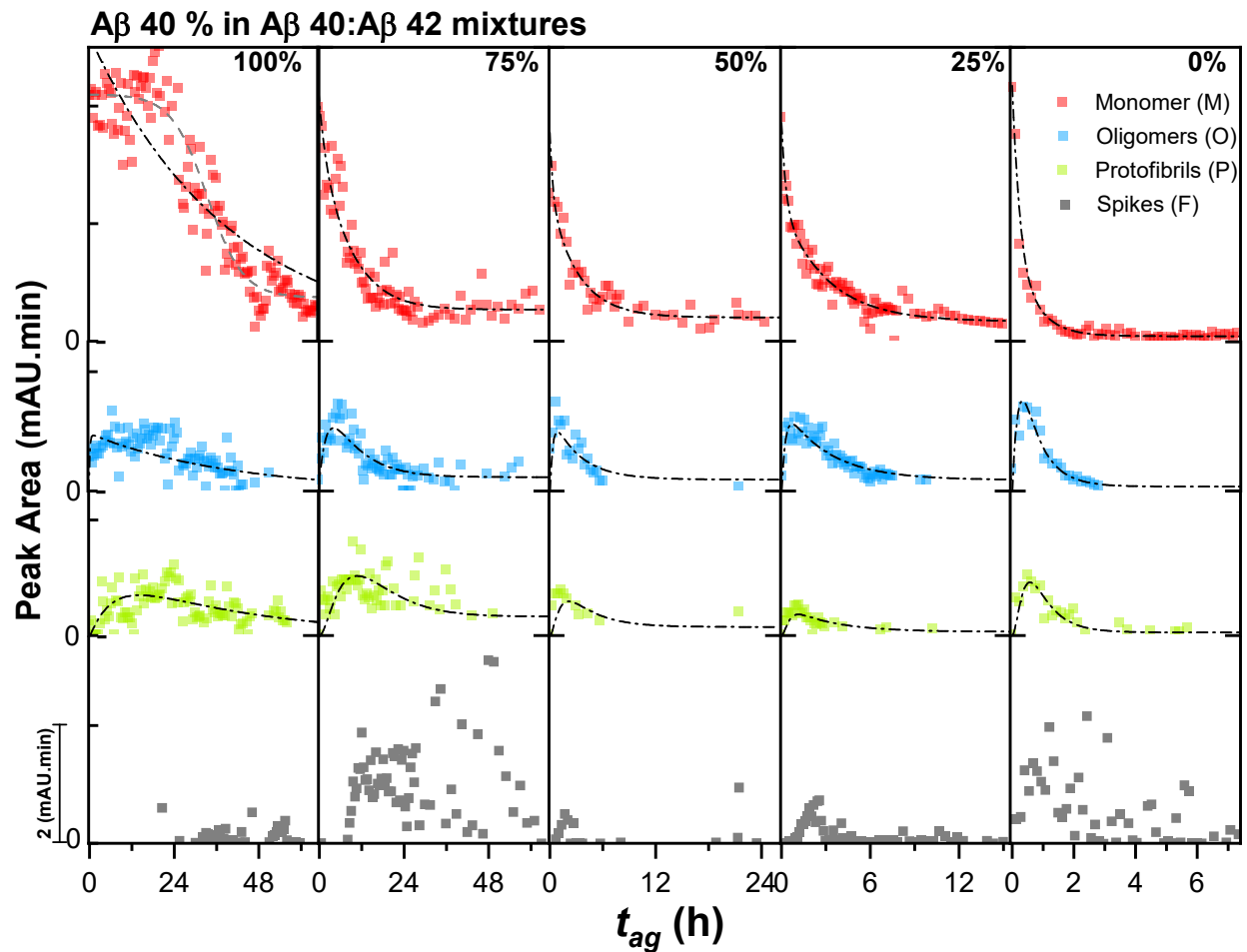
381 amyloid peptides, with potential applications to a wide range of biological, organic and inorganic
382 polymer systems.

383 **Table 1.** Dependence of various parameters on the proportion of A β (1-40) in the studied mixtures.
384 Populations' size: Average hydrodynamic radii of the monomer, oligomers and protofibrils size
385 populations obtained by the deconvolution of the taylorgrams with a finite number of Gaussian
386 functions. Kinetics parameters: values of the rate constants k obtained by fitting the peak areas
387 corresponding to the various populations with equations (1) to (3). The last two columns show the
388 time at which the oligomer population reaches its maximum, $t_{oligomers,max}$, and the initial
389 concentration of the monomer population, $M(t=0)$.

% A β (1-40) in A β (1-40):A β (1-42) mixtures	Populations' size						Kinetic parameters							
	Monomer and small oligomers		Higher mass oligomers		Protofibrils		Forward reactions			Backward reactions			$t_{oligomers,max} (h)$	$M(t=0) (mAU.min)$
	$\langle R_h \rangle$ (nm)	\pm SD	$\langle R_h \rangle$ (nm)	\pm SD	$\langle R_h \rangle$ (nm)	\pm SD	$k_{MO} (h^{-1})$	$k_{OP} (h^{-1})$	$k_{PF} (h^{-1})$	$k_{OM} (h^{-1})$	$k_{PM} (h^{-1})$	$k_{FM} (h^{-1})$		
100%	2.0	0.1	13.0	8.5	101.2	57.8	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>	<i>N.A.</i>
75%	2.4	0.2	9.3	1.8	140.2	43.7	1.106	0.817	0.943	1.497	0.123	0.050	4.171	3.551
50%	2.0	0.2	13.1	10.2	89.5	39.4	0.182	0.240	0.171	0.179	0	0.018	0.884	4.268
25%	2.0	0.2	17.9	10.4	135.6	56.9	1.457	1.226	2.190	1.386	1.332	0.045	0.722	3.709
0%	1.8	0.1	19.1	9.8	99.5	40.7	2.991	3.409	3.704	0	0.998	0.048	0.309	4.346



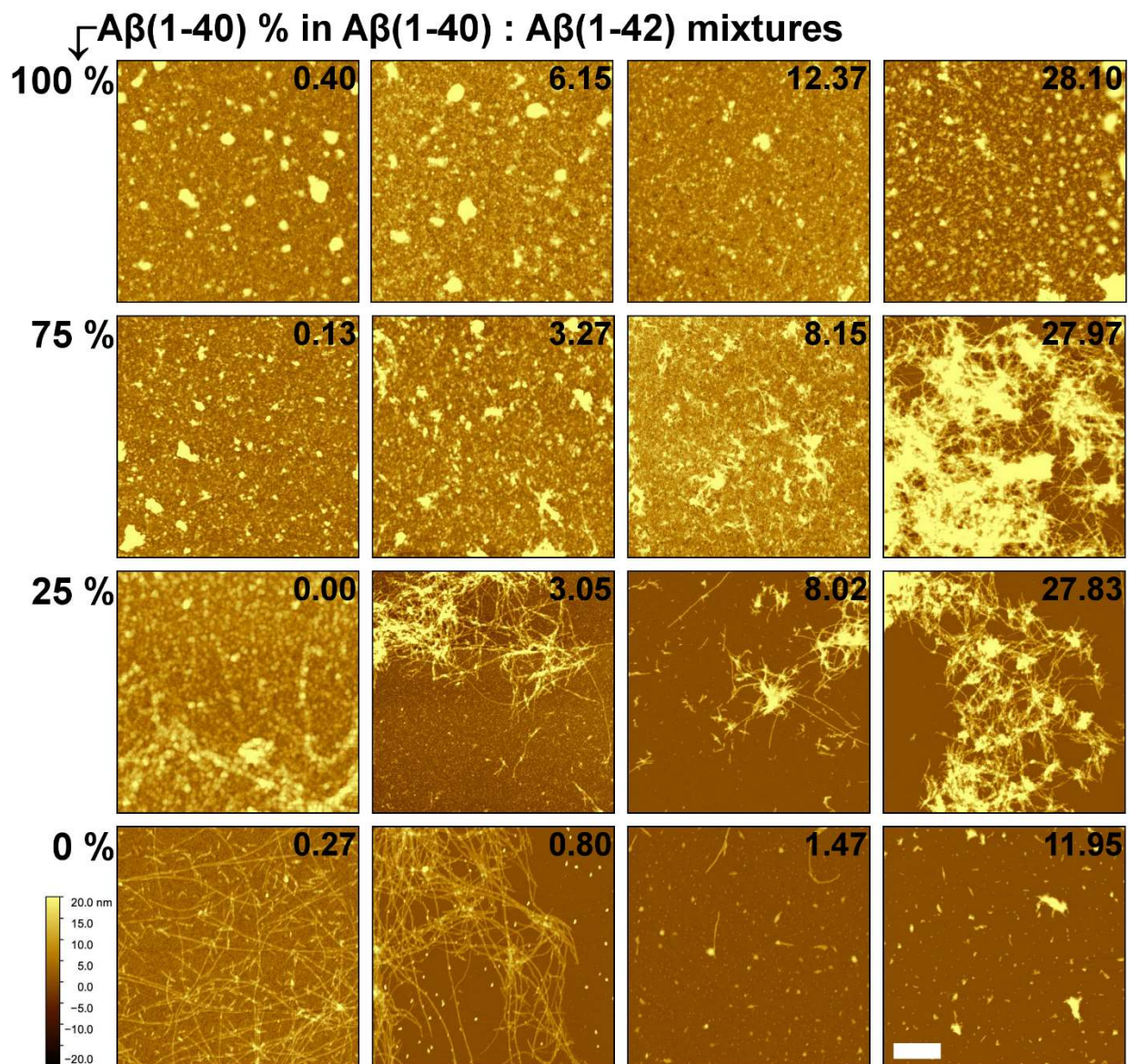
390
 391 **Figure 1.** Overview of selected Taylorgrams obtained during the aggregation process of A β (1-40)
 392 :A β (1-42) mixtures (A β (1-40) % of 100 %; 75%; 50%; 25% and 0%) at different common
 393 incubation times. Experimental conditions: Sample: 133 μ M of total peptide; 20 mM phosphate
 394 buffer, pH 7.4. Incubation: quiescent conditions at 37 $^{\circ}$ C. Fused silica capillaries: 50 μ m i.d. \times 40
 395 cm \times 31.5 cm. Mobile phase: 20 mM phosphate buffer, pH 7.4. Mobilization pressure: 100 mbar.
 396 Injection: 44 mbar for 3 s, $V_i \approx 7$ nL ($V_i / V_d \approx 1$ %). Analyses were performed at 37 $^{\circ}$ C. UV
 397 detection at 191 nm.



398

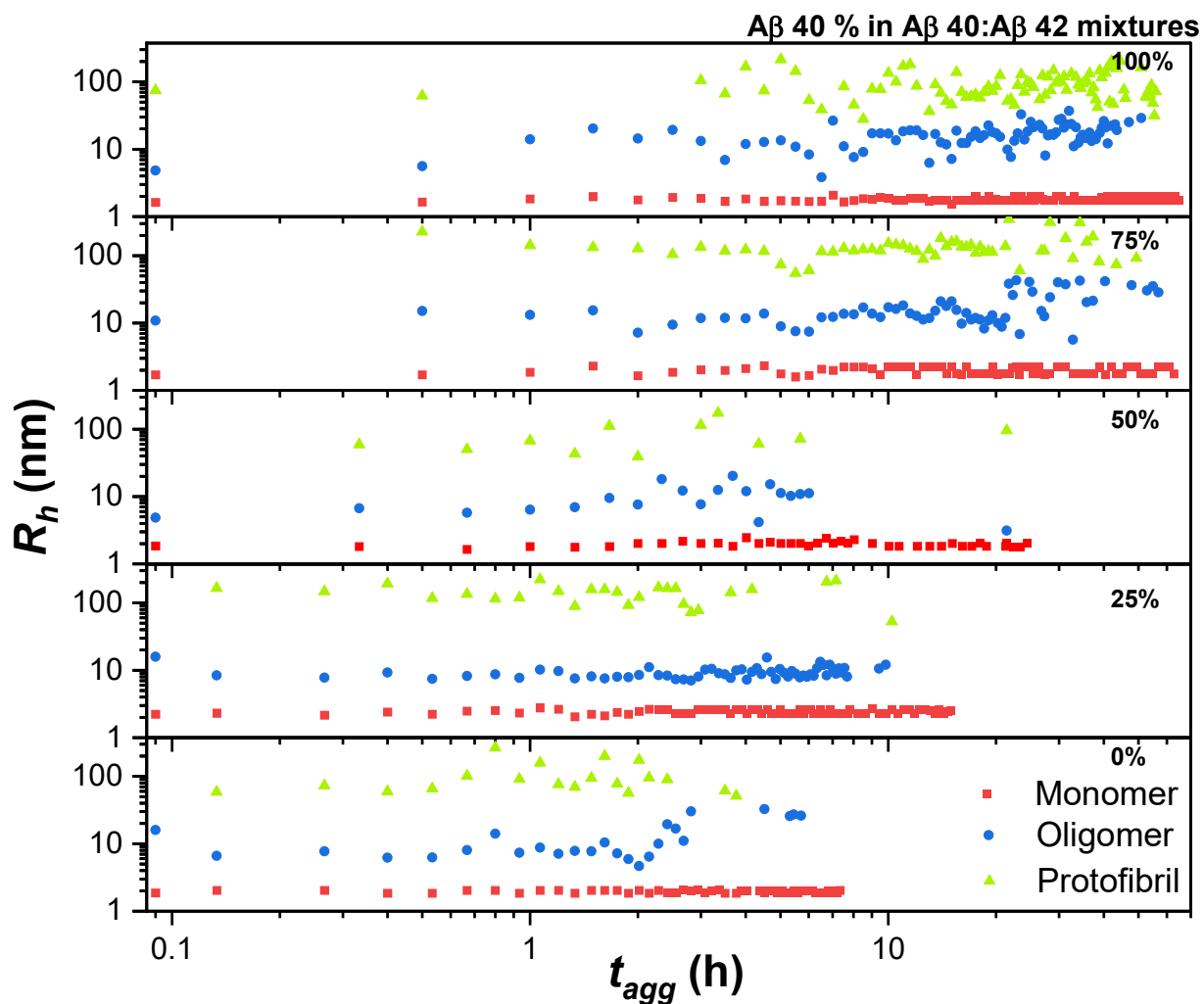
399 **Figure 2.** Peak area evolution of the different populations observed during the aggregation process
 400 of $A\beta(1-40):A\beta(1-42)$ mixtures and obtained using a fit with finite number of Gaussian functions.
 401 Three size populations are represented: monomer and low molar mass oligomers (■), higher molar
 402 mass oligomers (■), soluble protofibrils (■) and non-diffusing species (“spikes”) (■). Experimental
 403 conditions as in Figure 1. Black dashed-dotted lines are the kinetic fits obtained from equations
 404 (1) to (3). The grey dashed line in $A\beta(1-40)$ monomer distribution is a guide for the eye. The scale
 405 of the vertical axis is the same in all graphs and is indicated on the bottom-left corner of the figure.

406



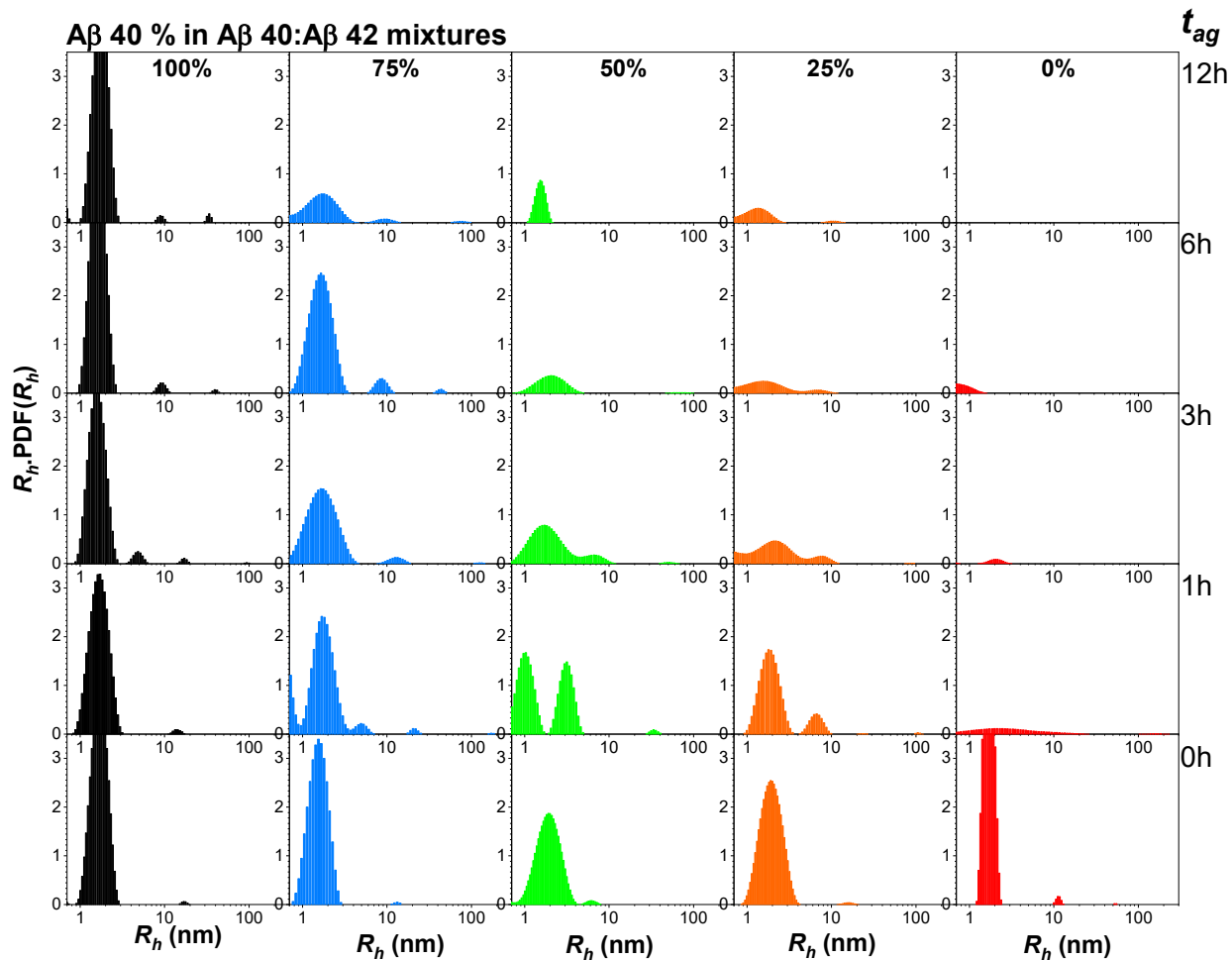
407

408 **Figure 3:** AFM images taken in intermittent contact mode under dry conditions, for different key
 409 times of the aggregation process in the presence of different peptide proportions. The $A\beta(1-$
 410 $40):A\beta(1-42)$ ratio is given on the left side of the panel, aggregation times in hours are overlaid
 411 on the images. The scale bar on the bottom right corner is valid for all images and equals 1 μm .



412

413 **Figure 4.** Hydrodynamic radius evolution of the different populations observed during the
 414 aggregation process of A β (1-40):A β (1-42) mixtures and obtained using a fit with finite number of
 415 Gaussian functions. Three size populations are represented: monomer and low molar mass
 416 oligomers $0.9 < R_h < 5$ nm (\blacksquare), higher molar mass oligomers $5 < R_h < 50$ nm (\bullet), and soluble
 417 protofibrils $50 < R_h < 300$ nm (\blacktriangle). Experimental conditions as in Figure 1.



418

419 **Figure 5.** Size distributions of A β (1-40):A β (1-42) mixtures obtained by CRLI analysis at selected
 420 incubation times $t_{ag} = 0$ to 12 h. Experimental taylorgrams corresponding to these distributions are
 421 shown in Figure 1, the experimental conditions are as in Figure 1.

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426

427 **AUTHOR CONTRIBUTIONS**

428 **Conceptualization** – HC and JC designed the experiments. **Investigation** – MD realized the TDA
429 experiments and treated the data. OD realized the AFM experiments. **Methodology** – LC and JC
430 fitted the data with the kinetic equations. **Software** – LC developed the script for the kinetic
431 modelling and the CONTIN TDA data treatment. **Supervision** – JC and HC supervised MD for
432 TDA experiments. JFH supervised MD for peptide synthesis. **Validation** – JC and HC validated
433 and interpreted the TDA results. OD and CB validated and interpreted the AFM results. **Writing**
434 – **original draft** – JC wrote the first draft and made the figures. OD made figure 3. **Writing** –
435 **review & editing** – All authors read and edited the manuscript. All authors have given approval
436 to the final version of the manuscript.

437

438 **Supporting information**

439 Theoretical aspects of TDA, experimental elution profiles, average hydrodynamic radii over the
440 whole aggregation process of each population and for all mixtures; CRLI analysis for all mixtures,
441 and the kinetic fits.

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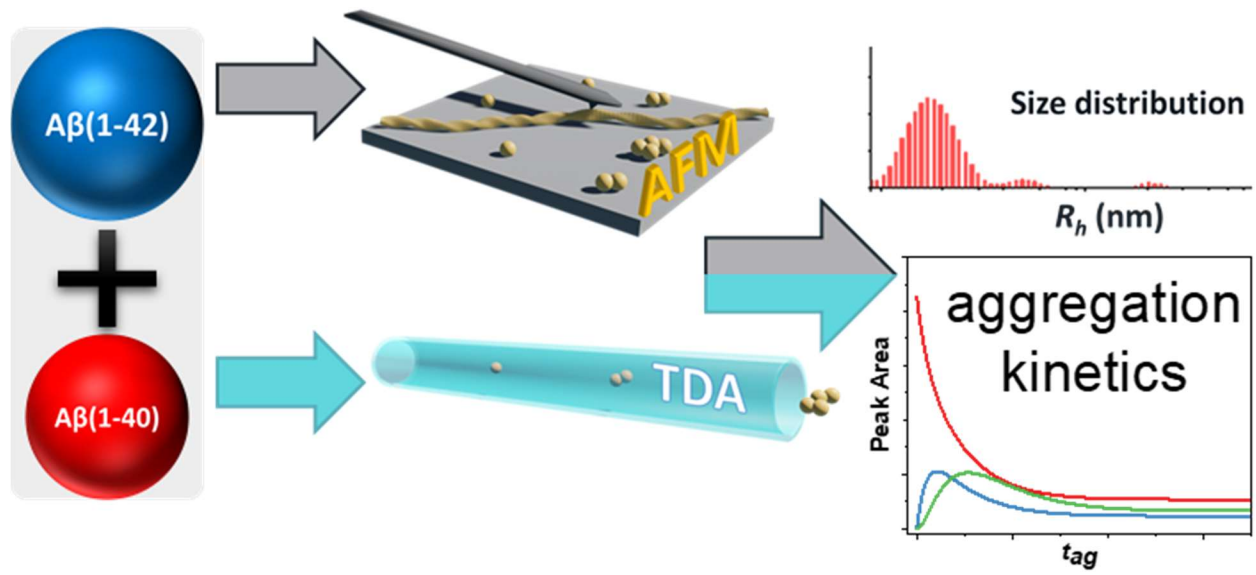
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