

## Fluorinated beta-sheet breaker peptides

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The aggregation of amyloid- $\beta$  peptide (A $\beta$ ) has been linked to the formation of neuritic plaques, which are pathological hallmarks of Alzheimer's disease. We synthesized peptides containing fluorinated amino acids and studied their effect on the A $\beta$  aggregation. The peptides were based on the sequence LVFFD, in which valine was substituted by either 4,4,4-trifluorovaline or 4-fluoroproline, or the phenylalanine at position 3 was replaced by 3,4,5-trifluorophenylalanine. Our results demonstrate that fluorination of the hydrophobic residue valine or phenylalanine is effective in preventing the A $\beta$  aggregation. This study opens up the possibility of using new sequences based on fluorinated amino acids to inhibit the amyloid-fibril formation.

## Introduction

Amyloid  $\beta$ -peptide (A $\beta$ ) is about 4 kDa and it accumulates extracellularly in the brain as amyloid fibrils, which form the dense aggregates – neuritic plaques – that pathologically characterize Alzheimer's disease (AD).<sup>1</sup> The aggregation process of A $\beta$  is described as a nucleation process, with the formation of unstable intermediates that act as seeds. At least two intermediates have been identified during amyloid fibril formation: intermediates that are soluble and of low molecular weight, composed of dimeric or oligomeric peptide molecules, and the protofibrils that are short and flexible fibrils.<sup>2</sup>

Increasing evidence suggests that soluble A $\beta$  oligomers might be critical for the onset of AD.<sup>3–7</sup> In the view of this hypothesis, a possible strategy to treat the disease is to inhibit the early stages of A $\beta$  aggregation.<sup>8</sup> The two hydrophobic cores of A $\beta$ , the residues 17–21 and 30–42, have been associated with the peptide aggregation.<sup>9,10</sup> Soto *et al.* designed a short peptide, based on the 17–21 hydrophobic core of A $\beta$  (LVFFA), which binds to the full length A $\beta$  and prevents its aggregation.<sup>11</sup> This inhibitor has a similar degree of hydrophobicity to that of the 17–21 core but it has a very low propensity to adopt a  $\beta$ -sheet conformation due to the presence of a proline residue. Valine, which is considered a key residue for the  $\beta$ -sheet formation, was replaced by proline and alanine was substituted by aspartic acid to improve the peptide solubility.<sup>11–13</sup> The sequence LPFFD,

referred to as iA $\beta_5$ , binds to A $\beta$  and blocks the A $\beta$ –A $\beta$  molecule interactions, inhibiting the formation of amyloid fibrils. Certain fluorinated compounds have also been proposed as inhibitors of the A $\beta$  aggregation.<sup>14–16</sup> Fluorinated solvents such as hexafluoroisopropanol induce an  $\alpha$ -helix structure in A $\beta$  peptide. A similar effect is observed when A $\beta$  interacts with poly(tetrafluoroethylene) surfaces or fluorinated nanoparticles.<sup>17–19</sup> Since the hydrogenated analogues of the nanoparticles were not able to prevent the amyloid fibril formation, it has been postulated that fluorine atoms play an important role in the inhibition of A $\beta$  aggregation. We have thus synthesized peptides containing fluorinated amino acids and studied their influence on A $\beta$  oligomerization. The fluorinated peptides are also based on the hydrophobic central residues of A $\beta_{(1–42)}$  17–21 (LVFFA) and on the iA $\beta_5$  beta-sheet breaker peptide. The aim is to study the impact of fluorine atoms on the beta-sheet breaker property of short peptide sequences and to understand the role of fluorine atoms on the A $\beta_{(1–42)}$  aggregation. The fluorinated sequences were covalently linked to polyethylene glycol (PEG) to increase their solubility (Fig. 1). We previously showed that coupling iA $\beta_5$  to PEG does not compromise its affinity to A $\beta$  or the iA $\beta_5$  beta-sheet breaker activity.<sup>20</sup> PEG has relatively low toxicity and is known to reduce proteolytic degradation.<sup>21</sup> The use of non-natural residues such as fluorinated amino acids is also described to efficiently improve the stability of peptides or proteins against enzymatic degradation.<sup>22</sup> The conjugates of fluorinated peptide–PEG were characterized by matrix-assisted laser desorption/ionization mass spectrometry. The kinetics of A $\beta_{(1–42)}$  aggregation, in the presence of the conjugates, were evaluated by the thioflavin T assay and the data were fitted using the crystallization-like model recently described by Crespo *et al.*<sup>23</sup> The ultrastructure characterization of the A $\beta$  aggregates was performed by transmission electron microscopy.

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**Amyloid-beta peptide**

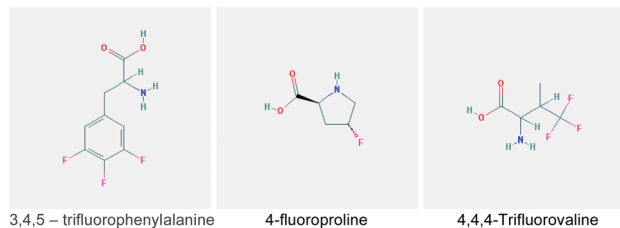
Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

**Conjugates**

Leu-FluoroPro-Phe-Phe-Asp-PEG (**LPFFD-PEG**)

Leu-Val-FluoroPhe-Phe-Asp-PEG (**LVFFD-PEG**)

Leu-FluoroVal-Phe-Phe-Asp-PEG (**LVFFD-PEG**)

**Fluorinated amino acids**

**Fig. 1** Sequences of the amyloid- $\beta$  peptide (1–42) and the fluorinated peptides conjugated to polyethylene glycol (PEG) used in this study. The chemical structure of the fluorinated amino acids is also shown (source: PubChem of the National Center for Biotechnology Information).

## Material and methods

### Synthesis of conjugates of fluorinated peptides and polyethylene glycol

The conjugates were prepared by the solid-phase supported strategy. Direct synthesis of the conjugate was performed applying a resin comprising a cleavable PEG spacer. Automated stepwise amino acid attachment was applied, following standard Fmoc-protocols. TentaGel PEG Attached Peptide resin (loading: 0.24 mmol g<sup>-1</sup>; Mn = 3200, PDI = 1.06 [GPC (THF, calibrated against linear PEG3200 standards, PSS, Germany)]) was purchased from Rapp, Polymere GmbH. Fmoc amino acid derivatives (Fmoc-phenylalanine OH, Fmoc-leucine OH, Fmoc-aspartic acid OH, Fmoc-valine OH, Fmoc-4,4,4-trifluoro-D,L-valine, Fmoc-3,4,5-trifluoro-L-phenylalanine and Fmoc-*trans*-4-fluoro-L-proline) were used as purchased from AnaSpec. The sequences of the fluorinated peptides are shown in Fig. 1. After the synthesis, the conjugates were dissolved in distilled water with 1% guanidinium hydrochloride and the pH was adjusted to 7 with 1 M sodium hydroxide. The samples were dialyzed against ultrapure water using a regenerate cellulose membrane (MWCO 1000 Da) for 4 days followed by lyophilization.

### Stock solutions of amyloid- $\beta$ peptide

A $\beta$ <sub>(1–42)</sub> (amyloid- $\beta$  peptide 1–42, purity > 95.22%, MW: 4514.14, Selleck Chemicals) was dissolved in HFIP (1,1,1,3,3,3-hexafluoro-2-propanol,  $\geq 99.8\%$ , Sigma-Aldrich) at a concentration of 1.0 mg mL<sup>-1</sup>. HFIP was evaporated with nitrogen flow and the peptide film was dissolved in DMSO (dimethyl sulfoxide for molecular biology,  $\geq 99.9\%$ , FW: 78.13, Sigma-Aldrich) at a concentration of 9.0 mg mL<sup>-1</sup>.

### Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF)

MALDI-TOF-MS measurements were performed using a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perceptive Biosystems, Inc., Framingham, MA, USA) at an acceleration voltage of 20 kV. The fluorinated peptide-PEG conjugates were dissolved in 0.1% TFA in acetonitrile-water (1 : 1, v/v) at a concentration of 0.1 mg mL<sup>-1</sup>. One microliter of the analyte solution was mixed with 1  $\mu$ L of an alpha-cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of the matrix dissolved in 1 mL of 0.1% TFA in acetonitrile-water (1 : 1, v/v). From the resulting mixture, 1  $\mu$ L was applied to the sample plate. Samples were air-dried at room temperature (25 °C). Each spectrum is a mean of 250 laser shots.

### Thioflavin T (ThT) binding assay

For kinetic studies, A $\beta$ <sub>(1–42)</sub> peptide (12.5  $\mu$ M) was incubated at 37 °C in 96 well plates (Nunc Delta Surface) with the fluorinated peptide-PEG conjugates (250  $\mu$ M) in the presence of ThT (0.7 mg mL<sup>-1</sup>) in a PBS buffer. The ThT solution was filtered using a 0.2  $\mu$ m syringe filter before adding to the A $\beta$  samples. The fibrils conjugated with ThT have the excitation maximum at 450 nm and enhanced emission at 482 nm.<sup>24</sup> The fluorescence intensity was measured every 30 minutes during 24 hours using a Biotek Synergy 2 fluorescence spectrometer with the excitation filter at 420/50 nm and the emission filter at 485/20 nm. Student's *t*-test statistical analysis was used to determine statistical significance between A $\beta$ <sub>(1–42)</sub> incubated alone and A $\beta$ <sub>(1–42)</sub> incubated with conjugates ( $n \geq 3$ ).

### Theoretical crystallization-like model

The crystallization-like model (CLM) is a generic two-parameter model that describes protein aggregation kinetics by a sequence of nucleation and growth steps. The CLM is represented by the following equation, in which  $\alpha$  represents the normalized fraction of amyloid protein converted into fibrils.

$$\alpha = 1 - \frac{1}{k_b[\exp(k_a t) - 1] + 1} \quad (1)$$

and  $k_a$  and  $k_b$  are the growth and the nucleation-to-growth rate constants. The two parameters are related to the time required to reach half of the total fibril conversion ( $t_{50}$ ) and to the aggregation rate ( $v_{50}$ ) given the slope of the  $\alpha(t)$  curve at that instant.

### Transmission electron microscopy (TEM)

The A $\beta$ <sub>(1–42)</sub> peptide (100  $\mu$ M) was incubated at 37 °C with each of the fluorinated peptide-PEG conjugates (2 mM) in a PBS buffer (10 mM, pH: 7.4), for 48 hours. An aliquot of each sample (5  $\mu$ L) was placed on carbon-formvar coated 200–400 mesh spacing grids and let to adsorb for five minutes. The negative staining was performed with a 2% filtered aqueous solution of uranyl acetate for 45 seconds. The grids were visualized using a Jeol JEM 1400 electron microscope at 80 kV.



## Results

### Fluorinated peptide-PEG conjugates

The fluorinated sequences conjugated to PEG were analysed by MALDI-TOF-MS. The spectrum of LP<sub>F</sub>FD-PEG is depicted as representative of the results acquired for the conjugates and shows the typical distribution of the polymer PEG with the characteristic repeat unit of  $44.1 \pm 0.5$  Da, which could be assigned to the ethylene oxide (EO) monomer of the PEG (Fig. 2). The experimentally found signal at  $m/z$  3694.8, for example, can be assigned to  $[M + K] = 3692.3$  Da by assuming an  $M_{[EO]}$  of 44.05 Da,  $n = 68$  units, a mass of the peptide  $M_{[peptide]} = 655.8$  Da, a mass of the polymer end groups  $1H = 1$  Da and the mass of a potassium counter ion  $M_{[counter\ ion]} = 39.1$  Da.

### Impact of the peptide-PEG conjugates on A $\beta_{(1-42)}$ fibrillation

The aggregation of A $\beta_{(1-42)}$  incubated at 37 °C with LP<sub>F</sub>FD-PEG, LV<sub>F</sub>FD-PEG and LV<sub>F</sub>FD-PEG at a molar ratio of 1 : 20 was evaluated by the ThT binding assay (Fig. 3 and 4). At 15 minutes incubation time, the ThT fluorescence intensity is low for all samples, which indicates low content of amyloid fibrils. After two hours, a large increase in the fluorescence intensity is observed for A $\beta_{(1-42)}$  alone and for the A $\beta_{(1-42)}$ :LP<sub>F</sub>FD-PEG samples, reaching the maximum at 4 hour incubation time (the fluorescence intensities were normalized to that of A $\beta_{(1-42)}$  incubated with ThT at 37 °C for 24 hours). The fluorescence of ThT in the presence of A $\beta_{(1-42)}$  and LV<sub>F</sub>FD-PEG showed only a moderated increase (53%) after 4 hours, whereas that of the sample A $\beta_{(1-42)}$ :LV<sub>F</sub>FD-PEG remained low (15%). The conjugates in the absence of A $\beta_{(1-42)}$  do not induce any change in the fluorescence signal of ThT.

The kinetic data were fitted according to the recently proposed CLM model (Fig. 4). The time at half fibril conversion ( $t_{50}$ ) and the aggregation rate ( $v_{50}$ ) were obtained by fitting a sigmoidal function to each kinetic trace according to the CLM (Table 1).<sup>23</sup> In the case of sigmoidal-type aggregation kinetics such as those represented in Fig. 4,  $v_{50}$  is solely dictated by the growth rate constant  $k_a$ . The parameter  $v_{50}$  did not change significantly in the presence of the conjugates. A marked increase in the value of  $t_{50}$  is, however, evident for the

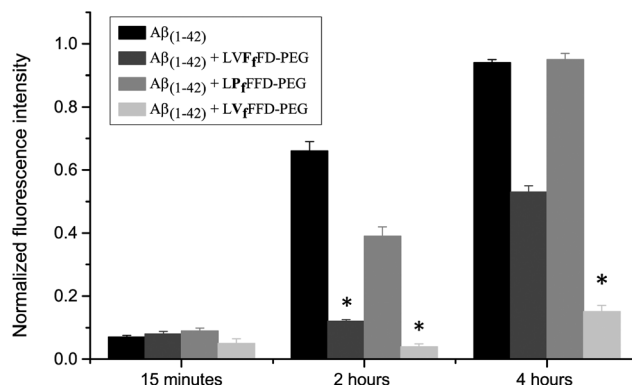


Fig. 3 Fluorescence intensity of thioflavin T in A $\beta_{(1-42)}$  samples containing fluorinated peptide-PEG conjugates incubated at 37 °C. The fluorescence signal was normalized to the maximum intensity of the sample of A $\beta_{(1-42)}$  without conjugates. \*Significantly different from the A $\beta_{(1-42)}$  control sample ( $p < 0.01$ ).

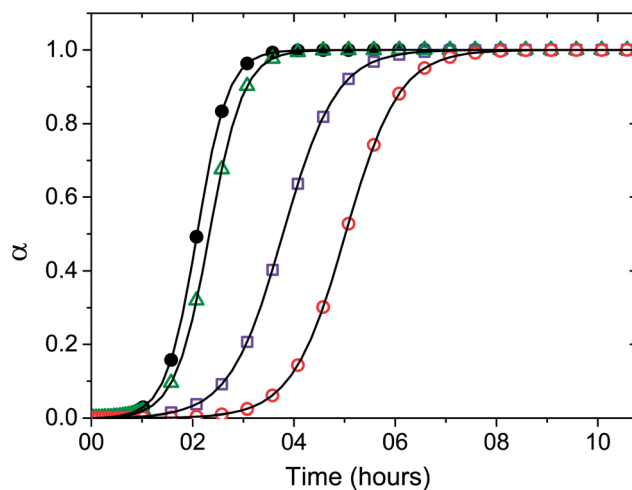


Fig. 4 Numerical fit (solid lines) of eqn (1) to the kinetic results of the A $\beta_{(1-42)}$  peptide incubated at 37 °C in PBS buffer in the absence (●) and in the presence of LP<sub>F</sub>FD-PEG (Δ), LV<sub>F</sub>FD-PEG (○), and LV<sub>F</sub>FD-PEG (□) conjugates, monitored by ThT fluorescence. The A $\beta_{(1-42)}$  concentration was kept constant at 12.5 μM and the molar ratio of A $\beta_{(1-42)}$  : conjugates was 1 : 20. The aggregation rate ( $v_{50}$ ) was obtained by fitting a sigmoidal function to each kinetic trace according to eqn (1).

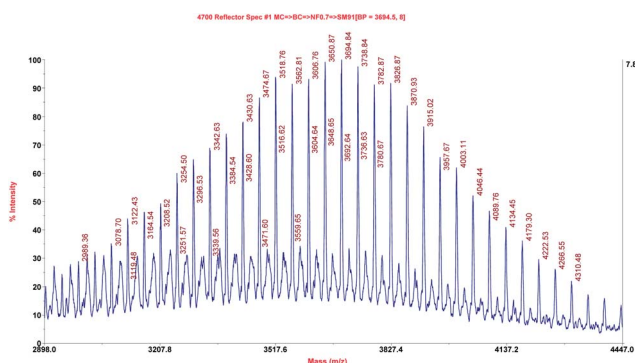


Fig. 2 MALDI-TOF-MS of a LP<sub>F</sub>FD-PEG conjugate.

LV<sub>F</sub>FD-PEG and LV<sub>F</sub>FD-PEG, which can be explained by the CLM as the result of either nucleation-prevention or solubility-change effects. The latter hypothesis is discarded by the fact that the maximum fluorescence signal did not show significant variation in the presence of conjugates. Therefore, we ascribe the increased induction times to the inhibition of the nucleation step as a possible consequence of the stabilization of the A $\beta$  molecule by the conjugates. The higher value of the induction time  $t_{50}$  is obtained for the conjugate containing the fluorinated valine (LV<sub>F</sub>FD-PEG), suggesting a stronger inhibitory effect in this case. Inhibitors of the amyloid fibril formation may act upon the initial assembly of macromolecules forming the stable nuclei (nucleation step) or block the subsequent addition





**Table 1** Time required to reach half of the total fibril conversion ( $t_{50}$ ) and delay in the aggregation of  $A\beta_{(1-42)}$  in the absence and the presence of fluorinated peptide conjugates and LPFFD sequence

	$t_{50}$ (hours)	Delay in aggregation (hours)
$A\beta_{(1-42)}$ alone	2.1	—
$A\beta_{(1-42)}$ and LPFFD-PEG	2.4	0.2
$A\beta_{(1-42)}$ and LVFFD-PEG	3.6	1.5
$A\beta_{(1-42)}$ and LVFFD-PEG	5.2	3.1
$A\beta_{(1-42)}$ and $iA\beta_5$	5.4	3.3

of new growth units (growth step).<sup>23</sup> A third way of action involves the alteration of the thermodynamic solubility of the polypeptide. This alternative is more unlikely to result *in vivo* since cell and tissue media are in general buffered against non-specific interactions. Compared to the effect of a well-known and well accepted beta-sheet breaker peptide,  $iA\beta_5$  (LPFFD), on  $A\beta_{(1-42)}$  aggregation, a similar effect was obtained by the LVFFD-PEG conjugate (3.1 hours) (Table 1). At the same molar ratio and with the same  $A\beta_{(1-42)}$  batch, the reduction of the aggregation time of  $A\beta_{(1-42)}$  is similar for  $iA\beta_5$  and LVFFD-PEG. The conjugate containing fluorinated phenylalanine (LVFFD-PEG) induced an inhibitory effect of 1.5 hours and the LPFFD-PEG did not inhibit the  $A\beta_{(1-42)}$  aggregation.

The  $A\beta_{(1-42)}$  : conjugate molar ratio of 1 : 5 did not significantly change the aggregation parameters of  $A\beta$ , which indicates that there is a threshold concentration for the  $A\beta$ -conjugate interactions.

TEM analysis showed that  $A\beta_{(1-42)}$  incubation at 37 °C for 48 hours resulted in amyloid-like, unbranched fibrils (Fig. 5).  $A\beta_{(1-42)}$  also formed fibrils in the presence of the conjugates (or  $iA\beta_5$ ), although the samples containing LVFFD-PEG and LVFFD-PEG show also small aggregates. This analysis

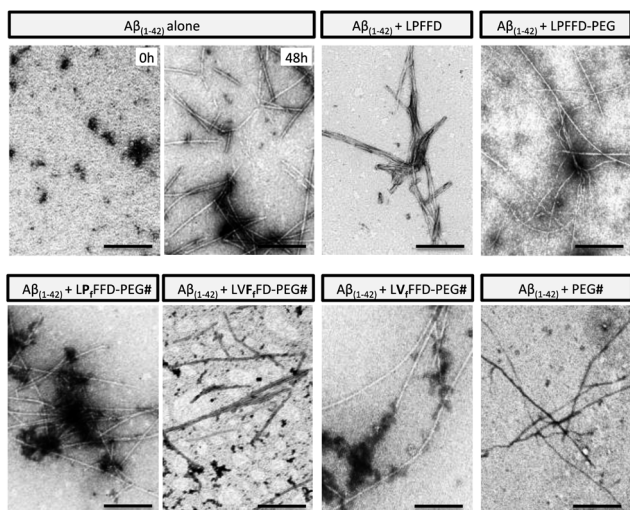
demonstrates that the bioconjugates and the beta-sheet breaker  $iA\beta_5$  are only delaying the  $A\beta_{(1-42)}$  fibrillogenesis.

## Discussion

$A\beta_{(1-42)}$  is an amphipathic peptide, which self-associates into oligomers that are toxic to cells.<sup>25</sup> The peptide aggregation eventually leads to the formation of amyloid fibrils, which are known to bind to Congo red and thioflavin T dyes. The oligomerization and aggregation of  $A\beta_{(1-42)}$  are difficult to control and although many attempts have been made to find a way to inhibit these processes, the understanding of how different factors can contribute to their initiation is still puzzling. The study on the self-assembly and structural transformation of other amyloid-fibril-forming peptides will provide important insights into the understanding of the fibril formation process and to find ways to prevent it.<sup>26–28</sup>

We relied on the knowledge that fluorine atoms seem to play a role in the prevention of  $A\beta_{(1-42)}$  aggregation and developed peptides with fluorinated amino acids to screen for fibrillization inhibitors. The ThT assay evidenced that two of the fluorinated peptides (LVFFD-PEG and LVFFD-PEG) are able to delay the aggregation of the  $A\beta_{(1-42)}$ . Hydrophobic interactions and H-bonding among side-chain groups are the two major driving forces that control protein aggregation.<sup>29</sup> A large amount of evidence indicates that there are three distinct regions within the  $A\beta_{(1-42)}$  sequence that might be involved in its aggregation: the central hydrophobic cluster corresponding to the 17–21 region, the 23–28 residues and the hydrophobic C-terminus.<sup>30–32</sup> Considering that fluorine atoms increase the hydrophobicity of the sequences, it is expected that they would interact with the hydrophobic regions of  $A\beta_{(1-42)}$  to be segregated away from the water. This interaction might contribute to the prevention of  $A\beta$  molecules to interact with each other, thus inhibiting their aggregation. The PEG molecules linked to the fluorinated peptides can also contribute to the interactions of the conjugates with  $A\beta_{(1-42)}$ . We have previously shown that the pegylation of a beta-sheet breaker peptide did not significantly change its binding to  $A\beta_{(1-42)}$ .<sup>29</sup> PEG is described to bind to proteins such as albumin and lysozyme even in solution with an ionic strength similar to the physiological conditions.<sup>33</sup> In the case of the fluorinated peptide-PEG conjugates, their effect on  $A\beta_{(1-42)}$  cannot be simply explained by PEG and  $A\beta$  interactions since the three conjugates show different outcomes.

The fluorinated amino acids of the conjugates may play an important role in delaying the  $A\beta_{(1-42)}$  aggregation by interacting with the hydrophobic residues of the peptide. There is also a geometric preference for the interactions between the C-F bond and the side-chain amides of glutamine (E) 15 and asparagine (N) 27 residues.<sup>34</sup> Therefore, the fluorine atoms might be able to interact with the two stretches of  $A\beta$  hydrophobic residues responsible for their oligomerization (17–21 and 30–42 residues) and/or with residues in their vicinity by exploiting the same interactions as in the  $A\beta_{(1-42)}$  assembly. A conformational tightened state is produced by the interplay of ionic and hydrophobic groups of both peptide and the conjugates.



**Fig. 5** TEM images of  $A\beta_{(1-42)}$  immediately after preparation (0 h) and incubation for 48 h at 37 °C in the absence and the presence of  $iA\beta_5$  (LPFFD), pegylated  $iA\beta_5$ , fluorinated peptide-PEG conjugates or PEG (molar ratio of 1 : 20). The scale bar corresponds to 200 nm.



Ferrão-Gonzales *et al.* showed that (1-anilino-8-naphthalene sulfonate)-derived molecules inhibit A $\beta$  aggregation due to their dual nature provided by hydrophobic regions and charged groups.<sup>35</sup> Vieira *et al.* demonstrated the effectiveness of different fluorinated alcohols in the  $\beta$ -to- $\alpha$  refolding process of A $\beta$ <sub>(1–40)</sub>.<sup>14</sup> The observed effect was interpreted as a result of alteration of the hydration shell of the peptides and hydrophobic effects of fluorine groups. Montserret *et al.* have shown that although the folding of amphipathic  $\alpha$ -helical peptides in hydrophilic SDS micellar solutions is mostly driven by electrostatic interactions, when hydrophobic peptides are considered, SDS-peptide hydrophobic interactions might be sufficiently strong to induce  $\alpha$ -helical-rich structures.<sup>36</sup> The effect of fluorinated complexes made of polyampholyte and the sodium salt of per-fluorododecanoic acid on A $\beta$  was attributed to the hydrophobic and acidic characters of the perfluorododecanoic acid.<sup>18,19</sup> We propose that LVF<sub>1</sub>FD-PEG and LV<sub>1</sub>FFD-PEG have the necessary hydrophobicity and spatial geometry to allow the interaction with A $\beta$ <sub>(1–42)</sub>. LP<sub>1</sub>FFD-PEG, with one fluorine atom in the proline residue, was not able to prevent A $\beta$ -A $\beta$  interactions. Proline residues are generally solvent-exposed in proteins and they are considered binding motifs in protein-protein interactions.<sup>37</sup> However it is possible that the presence of the fluorine atom in the proline residue of the LP<sub>1</sub>FFD-PEG prevents the exposure of the conjugate amino acids to A $\beta$ . Comparing the LP<sub>1</sub>FFD-PEG with the non-fluorinated analogue, one can assume that the fluorine atom increases the steric hindrance of the conjugate reducing the access to the proline residue. On the other hand the hydrophobic interactions between LP<sub>1</sub>FFD-PEG and A $\beta$  are probably not strong enough when compared to the A $\beta$ -A $\beta$  interactions. Apart from the apparently reduced interactions between A $\beta$  and LP<sub>1</sub>FFD-PEG, the position of fluorine atoms at the extremity of the conjugate and the fluorination of hydrophobic residues seem to favour the inhibitory effect of the sequences on A $\beta$  amyloid fibril formation.

## Conclusions

The effects of fluorinated alcohols, molecules or particles on A $\beta$  fibril formation are typically very different from those of their hydrogenated analogues. In general, it is possible to modify hydrogenated molecules or particles with fluorine atoms in such a way that they would be able to induce an  $\alpha$ -helix structure on A $\beta$ , a conformation which is less prone to aggregation. However, fluorination of molecules does not always lead to the inhibition of amyloid fibril formation but it can instead promote it. Therefore, it is important to understand the interactions of fluorinated molecules with A $\beta$  peptide. We propose the use of beta-sheet breaker peptides as model molecules to study the effect of fluorination on the A $\beta$  aggregation. Therefore, sequences that play an important role in A $\beta$  amyloid fibril formation were modified with fluorine atoms. Our work shows that fluorination of hydrophobic amino acids such as valine or phenylalanine of sequences that interfere with amyloid fibril formation induces a significant delay in the A $\beta$  aggregation process. This effect is likely a result of the interaction between the fluorinated amino acids and the hydrophobic residues of

A $\beta$ , which is strong enough to prevent the contact between A $\beta$  molecules and thus it prevents the peptide aggregation. The fluorination of other amino acids or the modification of the fluorinated sequences with hydrophilic residues at different positions will contribute to better understand the interactions between A $\beta$  and fluorinated peptides.

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