RSC Advances

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Please do not adjust margins

RSC Advances

COMMUNICATION

DMSO Affects $A\beta_{1-40}$'s Conformation and Interactions with Aggregation Inhibitors as Revealed by NMR

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

D. V. Laurents*[a], D. Pantoja-Uceda[a], L. C. López[b,c], J. A. Carrodeguas[b,c], M. Mompeán[a], M. Á. Jiménez[a], and J. Sancho*[b,c]

Abstract: We show via 3D-heteronuclear NMR spectroscopy that $A\beta_{1-40}$ adopts a disordered conformational ensemble with fluctuating turns in $DMSO_{d6}$. Using NMR, we map the binding sites of three water-insoluble aggregation inhibitors to $A\beta_{1-40}$ in $DMSO_{d6}$ and discover remarkable differences in $A\beta_{1-40}$ recognition by a fourth inhibitor in H_2O versus $DMSO_{d6}$.

Introduction: Alzheimer's disease is the most common neurodegenerative illness and a major cause of death in developed nations. Despite major efforts, no drugs that effectively prevent or cure the disease are currently available. Many biophysical and preclinical studies require the amyloid β peptide (Aβ) to be monomeric and unfolded at the start of the experiment. However, since Aβ has a strong tendency to oligomerize in aqueous solution, several protocols [1] [2] [3] based on inteins or Click Chemistry have been developed to ensure that it is unfolded and monomeric. However, these approaches require special materials and expertise. The simplest approach is to dissolve lyophilized Aβ in a small amount of organic solvent and then dilute it into aqueous buffer. Out of several organic solvents tested; namely, acetylnitrile, trifluoroethanol, HFIP, DMSO, dichloromethane, water+0.1% TFA, only DMSO and HFIP maintain Aβ in a monomer state without β-structure [4,5]. Whereas using TFE and HFIP to prepare Aβ stock solutions is a relatively common practice, it was shown that small quantities of TFE [6] or HFIP [7] like those present after dilution into aqueous buffer, actually accelerate β-structure formation and aggregation. Unlike fluorinated alcohols, DMSO is a polar aprotic solvent and does not seem to promote the formation of Aβ aggregates or β-structure, either neat or in any dilution with water [5] or alter Aβ aggregation kinetics [4]. Other studies of $A\beta_{1-40}$ showed it is monomeric in DMSO[8].

One aim here is to characterize the structure and dynamics of $A\beta_{(1-40)}$ DMSO using high field heteronuclear NMR spectroscopy to test its suitability for preparing stock solutions. One widely accepted hypotheses for Aβ neurotoxicity is that its small oligomers disrupt membrane function, leading to dendrite loss and altered neural signaling[9]. Moreover, Aβ oligomerization is thought to be stimulated by membrane components such as gangliosides[10]. Therefore, membrane-soluble Aβ inhibitors could have an advantage over water-soluble inhibitors. Recently, some of us [11] identified four compounds that block Aβ toxicity in cells. Three of the four are sparingly soluble in water, which thwarts attempts to characterize their binding to Aβ. However, they do dissolve well in DMSO, a polar aprotic solvent which partially mimics the membrane milieu. Like palmitoyloleoyl-phosphatidylcholine, a common neuron membrane component, DMSO can accept but not donate H-bonds. The second objective here is to characterize inhibitor binding to Aβ in DMSO using NMR. In the case of the water-soluble inhibitor, we shall compare how changing the solvent from water to DMSO affects its binding to Aβ.

Materials and Methods: $A\beta_{1-40}$, $^{13}C,^{15}N$-$A\beta_{1-40}$ and $DMSO_{d6}$ (99.9% atom D) were purchased from rPeptide and Aldrich, respectively. All NMR spectra were acquired at 30 ºC (in $DMSO_{d6}$) or 5ºC (aqueous solution) in solvent-matched Shigemi NMR tubes on a Bruker 800 MHz (^1H) spectrometer, equipped with a triple resonance cryoprobe and Z-gradients. The $^1H,^{13}C,^{15}N$ resonance assignments of $^{13}C,^{15}N$-$A\beta_{1-40}$ were obtained by analysis of a thorough series of (4,2)D, 3D and 2D NMR spectra as described more fully in the **Electronic Supplementary Information**. For the titration experiments, small volumes of inhibitor compounds, predissolved in solvent to a known concentration determined by weight, were added to the ^{15}N-$A\beta_{1-40}$ sample.

Results and Discussion: First we found that adding small amounts of $DMSO_{d6}$ to a ^{15}N-Aβ sample in aqueous buffer produced small,

[a.] Dr. D. V. Laurents, Dr. D. Pantoja-Uceda, Dr. M. A. Jiménez, Mr. M. Mompeán
Instituto de Química Física "Rocasolano", CSIC
Serrano 119, E-28006, Madrid, SPAIN
E-mail: dlaurents@iqfr.csic.es
[b.] Prof. Dr. J. Sancho, Dr. L. C. López, Dr. J. A. Carrodeguas
Joint Unit BIFI-IQFR, CSIC, Biocomputation & [c]Complex Systems Physics Institute (BIFI), Universidad de Zaragoza, Mariano Esquillar, Edificio I + D, E-50018, Zaragoza, SPAIN
Email: jsancho@unizar.es

* DVL & JS are both corresponding authors.
†Electronic Supplementary Information (ESI) available: Detailed experimental procedures, Tables listing NMR experiments, $A\beta_{1-40}$ $^1H,^{13}C,^{15}N$ chemical shifts in $DMSO_{d6}$ and H/D exchanges rates and eleven Sup. Figures. See DOI: 10.1039/x0xx00000x

RSC Advances Accepted Manuscript

Please do not adjust margins

linear changes in the ^{1}H-^{15}N HSQC spectra (**Sup. Fig. 1),** suggesting that small proportions of $DMSO_{d6}$ do not notably perturb Aβ's conformational ensemble. In contrast, the ^{1}H-^{15}N HSQC spectrum of $A\beta_{1\text{-}40}$ is strongly altered in neat $DMSO_{d6}$ (**Sup. Fig. 1**). This reflects possible conformational changes or stripping of H_2O from $A\beta_{1\text{-}40}$ or both. To assign $A\beta_{1\text{-}40}$ in $DMSO_{d6}$, we utilized a standard 3D approach based on intraresidual and sequential $^{13}C\alpha$ and $^{13}C\beta$ connectivities (**Sup. Fig. 2**). Next, the 3D (H)CC(CO)NH and 3D H(CCCO)NH and (4,2)D HN(COCA)NH spectra (**Sup Table 1**) were analyzed to corroborate the backbone assignments and to assign many side chain resonances. Overall, the assignment process was straightforward except for some nuclei in or adjacent to His residues which showed significant broadening or low intensity in some spectra. The resulting ^{1}H, ^{13}C, ^{15}N $A\beta_{1\text{-}40}$ chemical shift values (δ) are reported in **Sup Table 2** and have been deposited in the BMRB under accession number **25450**. The assigned ^{1}H-^{15}N spectrum of $A\beta_{1\text{-}40}$ in $DMSO_{d6}$ is shown in **Fig. 1**. Integration of the ^{1}H-^{15}N peaks revealed that H5, D7, S8, H13, H14 (not observed) and L17 have low intensity and/or broad peaks. These differences could be due to conformational heterogeneity or exchange with residual H_2O. The aliphatic $–CH_2$- and $–CH_3$- groups show little δ dispersion; this is consistent with a lack of preferred conformations (see the ^{1}H-^{13}C HSQC spectrum, **Sup. Fig 3**). The δ of $\varepsilon C^{1}H_3$ of Met 35 is 2.00 ppm, which indicates that the sulfur is reduced. This point is relevant as oxidation of Met35 is believed to inhibit Aβ aggregation [12]. The ^{1}HN and $^{1}H\alpha$ δ values determined here resemble those reported previously for $A\beta_{1\text{-}40}$ in 95% DMSO/5% dichloroacetic acid [13] and $A\beta_{1\text{-}28}$ in neat DMSO [14] (**Sup. Fig. 4**).

Based on the obtained ^{15}N, $^{1}H\alpha$, ^{13}CO, $^{13}C\alpha$ and $^{13}C\beta$ assignments, the suite TALOS-N was used to predict tendencies to adopt secondary structure. Most residues were predicted to lack preferences except residues I31, I32 and M35, which trend toward β-strand conformations. These predictions should be interpreted with caution as DMSO is known to produce large δ changes in ^{13}CO, $^{13}C\alpha$, $^{13}C\beta$ and ^{1}H nuclei of short unstructured peptides[15]. In contrast, many inter-residual ^{1}HN – ^{1}HN NOEs were observed; namely between residues: 2-3, 7-8, 11-12, 17-18, 21-24, 23-24, 24-25, 25-28, 27-28, 31-32, 31-33, 32-33, 36-40 and 37-39 which are evidence for turn or helix-like conformations (**Sup. Fig. 5**). These findings, obtained using a 800 MHz spectrometer and a 150 ms mixing time, resemble and extend the observations, by Sorimachi & Craik [14], of ^{1}HN – ^{1}HN NOE crosspeaks between residues 2-3, 3-4, 4-5, 11-12, 12-13, 22-23, 23-24, 24-25, 25-26 in the Aβ fragment (1-28) in experiments performed at 400 MHz with a 250 ms mixing time. The few, weak ^{1}H aliphatic – ^{1}H aromatic interresidual NOEs observed likely arises from transient contacts and no stable hydrophobic cluster is present (*data not shown*).

Additional experiments were done to see if the turn or helix-like conformations detected by the NOEs are transient and weak or long-lasting and stable. The heteronuclear NOE ratios, which provides information on the ps-ns local backbone dynamics of $A\beta_{1\text{-}40}$ in neat $DMSO_{d6}$, are shown in **Fig. 1 and Sup Fig 6 and 7**. Overall, the polypeptide appears to be flexible; all residues have values much lower than the theoretical limit of 0.85 for complete rigidity. The region composed of Arg5 – Gln15 appears to be modestly rigid, residues Lys 16 to Val 24 are more flexible, and the first 3 and the last 12 C-terminal residues are highly flexible. Notably the heteronuclear NOE ratio values of $A\beta_{1\text{-}40}$ in $DMSO_{d6}$ closely resemble those reported previously [16] for $A\beta_{1\text{-}40}$ freshly dissolved in aqueous buffer (**Sup Fig. 7).** To test for stable secondary structure, a ^{15}N-$A\beta_{1\text{-}40}$ sample in $DMSO_{d6}$ was diluted into D_2O buffer, the first fast ^{15}N-^{1}H SOFAST HMQC showed that only 5 resonances, corresponding to F4, L34, I31, I32 and V40 resisted exchange. These resonances are predicted [17] to have the slowest H/D exchange kinetics (**Supporting Table 3**). By monitoring peak intensity over time, the residues' exchange rates were determined and protection factors of 17, 7, 7, 4 and 2 for F4, L34, I31, I32 and V40, respectively, were calculated (**Sup. Fig. 8**). These very low factors (**Sup. Table 3**) are solid evidence that $A\beta_{1\text{-}40}$ in DMSO adopts no stable secondary structure. To test if $A\beta_{1\text{-}40}$ forms amyloid-like conformers in DMSO, we performed ThT fluorescence assays. No significant differences in ThT fluorescence are induced by $A\beta_{1\text{-}40}$ that had been incubated in DMSO (**Sup. Fig. 9A**). In contrast, $A\beta_{1\text{-}40}$ incubated in aqueous solution induces a hundred-fold increase in ThT fluorescence (**Sup. Fig. 9B**) indicating the formation of amyloid-like conformers.

We utilized our assignments of $A\beta_{1\text{-}40}$ in DMSO to study how solvent affects the peptide's interaction with four inhibitors. The chemical structures and the NMR spectra of the four inhibitors are shown in **Sup. Fig. 10**. The spectral resonances observed are consistent with their chemical structures and no significant amounts of impurities were detected.

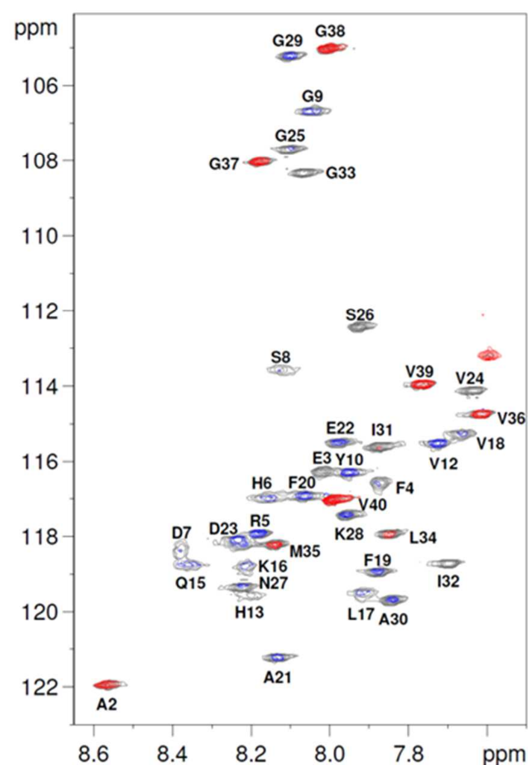

Figure 1. Superposition of ^{1}H-^{15}N HSQC Spectra of $A\beta_{1\text{-}40}$ in $DMSO_{d6}$ without (gray peaks) and with (red=negative / blue=positive peaks) application of the ^{1}H-^{15}N heteronuclear NOE. ^{1}H and ^{15}N chemical shifts are plotted on the x- and y-axes, respectively. The spectra are shown separately in **Sup. Fig. 6**.

RSC Advances Accepted Manuscript

Please do not adjust margins

We took advantage of C1´s relatively high water solubility to compare its binding to $A\beta_{1-40}$ in different solvent conditions. In aqueous solution, the ^1H-^{15}N HSQC spectra (**Sup. Fig. 11A**) show that regions most affected by C1 correspond to HNs belonging to the hydrophobic segments of $A\beta_{1-40}$ (**Fig. 2A**), which coincide more or less with the β-strands in mature Aβ amyloid fibrils [18]. In contrast, the ^1H-^{15}N HSQC spectra (**Sup. Fig. 11B**) reveal that in $DMSO_{d6}$, C1 binds to the HN groups of the 18 N-terminal residues of $A\beta_{1-40}$ (**Fig. 2B**), a region rich in polar and charged residues. This is an important result as it indicates, for the first time to our knowledge, that solvent can alter the binding mode of an inhibitor to Aβ. This finding has a crucial implication for developing therapeutics; namely, the Aβ binding sites of an inhibitor could well change according to the solvent milieu.

The effect of C2, C3 and C4 on Aβ´s ^{15}N-^1H resonances were also determined (**Sup. Fig. 12**). C2 appears to have a modest effect on Aβ's signals; only modest chemical shift changes in the first 10 residues are observed. In contrast, C3-induced ^{15}N-^1H chemical shift changes are large in magnitude and more extensive. Only residues 2, 3, 4, 12, 15, 18, 19, 29-34 and 37-40 and the sidechains of R5, Q15 and N27 are relatively unaffected by C3. The changes induced by C4 are somewhat larger than those provoked by C3. For all the samples, we attempted to further characterize the binding using NOESY spectroscopy, but no unambiguous NOEs could be assigned. This could be due to the low concentration of $A\beta_{1-40}$ (100 μM) and/or heterogeneity in the $A\beta_{1-40}$ / inhibitor complex. The basis for the inhibitors' divergent modes of interaction with $A\beta_{1-40}$ is not immediately apparent from their chemical structures [11]. C3 and C4, but not C2 contain a phenyl moiety, as does the recently discovered Aβ inhibitor D373[19]. This phenyl group might account for the more extensive interaction of C3 and C4 with $A\beta_{1-40}$ compared to C2 (**Sup Fig. 10**).

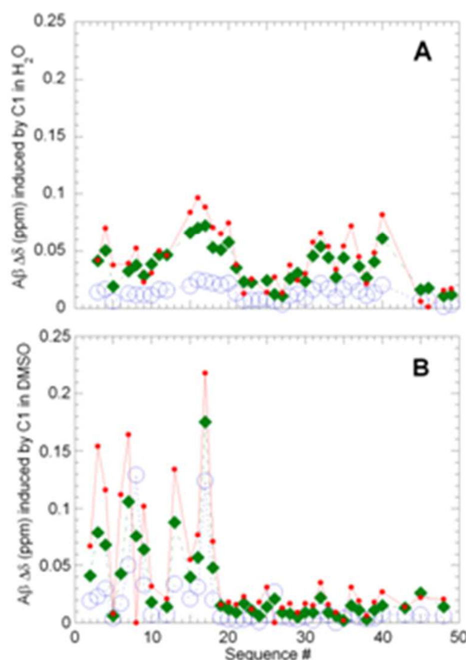

Figure 2. The weighted average shift changes for Aβ´s ^1H and ^{15}N nuclei in the presence of 1 eq of C1 (large open blue circles, dotted line), 3 eq C1 (green diamonds, dashed lines) and 5 eq C1 (small red circles) in water (**A**) and in $DMSO_{d6}$ (**B**). The data at sequence # 42, 45/46 and 48/49 correspond to the side chain groups of R5, Q15 and N27, respectively.

Conclusions

We have obtained for the first time the essentially complete ^1H, ^{13}C and ^{15}N resonance assignments of $A\beta_{1-40}$ in $DMSO_{d6}$. Utilizing these results, we determine that $A\beta_{1-40}$ adopts a disordered conformational ensemble in $DMSO_{d6}$ with only weak turn or helix-like structures. Since Aβ is monomeric [8] and chiefly unfolded without amyloid-like conformers (*this work*) in neat DMSO and because low concentrations of DMSO (which would be present after dilution into aqueous buffer) neither increase the rate of Aβ aggregation [5], nor significantly perturb the ^1H-^{15}N HSQC NMR spectrum of Aβ (*this work*), we conclude that dissolving Aβ in DMSO is a simple yet effective way to prepare unstructured stock solutions of Aβ. There is intense interest in developing small molecule therapeutics for AD [20], which is driven by the ever increasing number of patients and disappointing results from clinical trails for antibody-based drugs[21]. A key discovery here is that the binding of inhibitor compounds to $A\beta_{1-40}$ can be strongly affected by the solvent milieu. C1 binds to $A\beta_{1-40}$'s hydrophobic regions in aqueous buffer, but in $DMSO_{d6}$ it binds exclusively to the N-terminal residues. Based on this finding and considering that Aβ neurotoxicity likely occurs at the membrane[22], we emphasize the importance of testing potential $A\beta_{1-40}$ therapeutics in membrane-mimicking solvent media.

Notes and references

This work was supported by grants from the Governments of Spain (Project numbers CTQ2010-21567-C02-02, CTQ2011-22514, BFU2010-16297, SAF2013-49179-C2-1-R) and Aragon (B89-2011). We thank Profs. Marta Bruix and Avijit Chakrabartty for critical comments on the manuscript and Prof. Jorge Santoro for advice on NMR pulse sequences.

1. J. J. Bockhorn, K. L. Lazar, A. J. Gasser, L. M. Luther, I. M. Qahwash, N. Chopra, S. C. Meredith, *Peptide Sci.* **2009**, *94,* 511-520
2. A. Taniguchi, Y. Sohma, M. Kimura, T. Okada, K. Ikeda, Y. Hayashi, T. Kimura, S. Hirota, K. Matsuzaki, Y. Kiso, *J. Am. Chem. Soc.* **2006**, *128,* 696-697
3. Y. Sohma, H. Wang, A. Taniguchi, Y. Hirayama, T. Kakizawa, M. Yamaski, H. Mukai, Y. Kiso, *Bioorg. Med. Chem.* **2011**, *19,* 3787-37921.

RSC Advances Accepted Manuscript

Please do not adjust margins

4. S. Snyder, U. S. Ladror, W. S. Wade, G. T. Wang, L. W. Barret, E. D. Matayoshi, H. J. Huffaker, G. A. Krafft, T. F. Holzman, *Biophys. J.* **1994**, *67,* 1216-1228
5. C. L. Shen, R. M. Murphy, *Biophysical J.* **1995**, *69,* 640-651
6. Y. Fezoui, D. B. Teplow, *J. Biol. Chem.* **2002**, *277,* 36948-36954
7. M. R. Nichols, M. A. Moss, D. K. Reed, S. Cratic-McDaniel, J. H. Hoh, T. L. Rosenberry, *J. Biol. Chem.* **2005**, *280,*
8. W. Garzón-Rodríguez, M. Sepulveda-Becerra, S. Milton, C. Glabe, *J. Biol. Chem.* **1997**, *272,* 21037-21044
9. J. J. Palop, L. Mucke, *Nature Neuroscience* **2010**, *13,* 812-818
10. K. Yanagisawa, *J. Neurochem.* **2011**, *116,* 806-812
11. L. C. López, S. Dos-Reis, A. Espargaró, J. A. Carrodeguas, M. L. Maddelein, S. Ventura, J. Sancho, *J. Med. Chem.* **2012**, *55,* 9521-9530
12. L. Hou, H. Shao, Y. Zhang, H. Li, N. K. Memon, E. G. Neuhaus, J. M. Brewer, I.-J. L. Byeon, D. G. Ray, M. P. Vitek, *et al. J. Am. Chem. Soc.* **2004**, *126,* 1992-2005
13. N. A. Whittemore, R. Mishra, I. Kheterpal, A. D. Williams, R. Wetzel, E. H. Serpersu, *Biochemistry* **2005**, *44,* 4434-4441
14. K. Sorimachi, D. J. Craik, *Eur. J. Biochem.* **1994**, *219,* 237-251
15. K. Wüthrich, *NMR in Biological Research: Peptides and Proteins*. 1975, Amsterdam North Holland. .
16. R. Riek, P. Güntert, H. Döbeli, B. Wipf, K. Wüthrich, *Eur. J. Biochem.* **2001**, *268,* 5930-5936
17. Y. Bai, J. S. Milne, L. Mayne, S. W. Englander, *Proteins Struc. Func. Gen.* **1993**, *17,* 75-86
18. A. T. Petkova, Y. Ishii, J. J. Balbach, O. N. Antzutkin, R. D. Leapman, F. Delaglio, R. Tycko, *Proc Natl Acad Sci USA* **2002**, *99,* 16742-16747.
19. A. Fortner-McKoy, J. Chen, T. Schupbach, M. H. Hecht, *J. Biol. Chem.* **2012**, *287,* 38992-39000
20. A. J. Doig, P. Derreumaux, *Curr. Opin. Struct. Biol.* **2015**, *30,* 50-56
21. S. Salloway, R. Sperling, N. C. Fox, K. Blennow, et al., *Engl. J. Med.* **2014**, *370,* 322-333
22. G. M. Shankar, D. N. Walsh, *Mol. Neurodegeneration.* **2009**, *4,* 48

RSC Advances Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx